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Incidence and Growth of Some Pathogens in Freshwater Crayfish (Procambarus Clarkii, Girard).

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INCIDENCE AND GROWTH OF SOME PATHOGENS IN FRESHWATER CRAYFISH (PROCAMBARUS CLARKII GIRARD)

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by
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ABSTRACT

A study of the incidence of public health-related bacteria in live, unhandled, South Louisiana crayfish indicated that coliforms, E. coli, and fecal streptococci are normally found as contaminants in the product taken from natural sources. These organisms are also found in relatively high concentrations in the water from these sources. This indicates that these bacteria can possibly be carried over during the processing of the tailmeat and become a potential public health hazard. Coagulase-positive staphylococci, Shigella species, Salmonella species and C. botulinum were found not to be generally present in crayfish or the water from natural sources; consequently, if these organisms are present in the processed product, it would likely be the result of unsanitary processing conditions.

Growth curves showing short-time (175-hr) growth patterns and long-term storage (3-weeks) growth patterns of these public health-related organisms at 0, 5, 25 and 37°C revealed that none of the organisms grow well at refrigeration temperatures (5°C) in raw or cooked crayfish tailmeat and should not present a public health hazard if crayfish products are stored under proper refrigeration conditions. At 25 and 37°C all organisms appear to grow well in

raw tailmeat and significantly better in cooked tailmeat. In ice-pack the organisms decreased in number with storage time.

Up to 56 days ice storage (0°C) no type E toxin of C. botulinum was produced in raw or cooked crayfish tailmeat or in crayfish etouffé. At refrigeration temperature (5°C) type E toxin was produced in 33 days in all three products. At 30°C, toxin was detected in the raw tailmeat in 24 hr, while in the cooked tailmeat and the prepared product it was detected in 48 hr. At each temperature the product was spoiled by the time toxin was first detected.

There was a definite relation between pH of the product and inactivation of type E toxin. In raw and cooked tailmeat at 30° C toxin was inactivated when the pH reached 8.0 to 8.5. The pH of these products did not reach 8.0 at 0 and 5°C. The pH of etouffé decreased to 5.9 during storage at 30°C but inactivation of the toxin did not occur; however, when the pH of the etouffé was adjusted to 8.5 by the addition of NaOH the toxin was inactivated.

INTRODUCTION

Crayfish, which has also been commonly referred to as crawfish, crawcrab, crab, stonecrab crawdad, creekcrab, yabbie, mudbug, ecrevisses, or other local names, is found in fresh waters on all continents except Africa. There are more than 300 species found through the world, over 100 species in the United States, and 29 known species in Louisiana.

For many years trapping crayfish has been on a commercial scale. Various sources in Louisiana include swamps, creeks, ricefields, flooded basins, and control-flooded ponds. Daily catches are sold alive in retail markets or are processed in peeling plants and sold as fresh, peeled tailmeat.

The demand for crayfish and crayfish products has increased the size of this industry in recent years, however, sanitation and quality control measures have not been a primary concern in the industry. Crayfish come from a variety of sources and in many cases are handled under very casual conditions; consequently, the public health aspects of crayfish products are of significance. All crayfish used for this study were of the species Procambarus clarkii Girard.

Thus far, no controlled studies have been conducted on the microbiology of crayfish from South Louisiana sources, therefore this study was designed with the following objectives:

1. Conduct a survey to determine whether or not selected microorganisms of public health significance are natural contaminants of crayfish and if so in what relative proportions.

2. Analyze water samples to ascertain the degree of pollution of crayfish sources.

3. Determine growth patterns for these microorganisms under various storage conditions in raw and cooked crayfish tail flesh and in a prepared crayfish food product.

4. Study type E toxin production by Clostridium botulinum in raw and cooked crayfish tail flesh and in a prepared crayfish food product in relation to storage time, temperature, and pH.

LITERATURE REVIEW

Relatively few investigations offer pertinent information concerning the microbiology of crayfish. Far more is known about the diseases of living crayfish, particularly the crayfish pestilence, than its spoilage pattern of crayfish meat. Crayfish, consequently, offer a fertile field for bacteriological research, involving almost all phases of handling, processing and transportation.

Very little significant data are available which account for deteriorative changes causing spoilage in raw lobsters. There are, however, several indications that they follow the general pattern for crustaceans. The breakdown through autolysis and bacterial decomposition in broad terms was studied by Reed (1925) and Reed et al. (1929). Taking into consideration the extensive consumption of lobsters in both the United States and Europe, it is most surprising that almost no microbial studies are available that clarify the breakdown pattern of lobsters from the bacteriological or biochemical point of view. This might establish the general impression that these organisms possess good keeping characteristics. However, lobster spoilage as indicated by disagreeable odor, taste and flavor becomes so obvious that consumption ceases, thus this is undoubtedly a fruitful field for research.

The literature indicates that methods commonly utilized for the detection of fecal indicator organisms in water and milk are unsatisfactory when applied to foodstuffs (Larkin et al., 1956; Zaborawski et al., 1958; Kereluk and Gunderson, 1959). This holds true also for shellfish, where the same methods have been used for many years (Kelly, 1960). It is widely recognized that the Coliform Most Probable Number (MPN) count is limited in its usefulness because of the diverse origin of the organisms included in the coliform group. Also many coliforms are derived from nonhuman sources and are normally expected to be present in raw or manufactured food products. Considerable attention has been given therefore to the estimation of the so-called fecal organism, Escherichia coli type I. The E. coli (44.5°C) confirmation test of Hajna and Perry (1943) has been widely utilized to isolate this organism. The value of this test in assessing the sanitary quality of potable waters has been generally recognized and its extension to shellfish and frozen foods has been advocated.

An evaluation of the E. coli (44.5°C) confirmation test for estimation of E. coli type I as an index of sanitary quality of frozen seafoods (raw as well as precooked) and of processing raw material was made by Raj and Liston (1961b). They found 48 of 163 samples tested gave a positive E. coli test, but only 16 samples actually contained fecal E. coli, and a total of 143 E. coli gas positive cultures failed to give characteristic colonies on eosin-methylene

blue (EMB) agar plates. It has also been shown that the production of a characteristic metallic sheen on EMB agar medium is not a reliable criterion for isolating fecal E. coli.

Berry (1941) showed that oyster meats that had been macerated, yielded higher bacterial counts than whole specimens, and the count obtained was directly proportional to the degree of disintegration. The count of coliform bacteria was also increased.

Hunter and Harrison (1928) stated that in shucked oysters stored at temperatures below 10°C, no multiplication of E. coli or other lactose-fermenting bacteria occurred. Tonney and White (1926), on the other hand, reported that E. coli did multiply at 5 to 8°C. Wilson and McCleskey (1951b) could not confirm this, but found that coliform organisms readily developed when shucked oysters were stored at 4 to 6°C. Initially, the increase was slow, but enormous numbers were encountered after 3 to 4 weeks of storage. These workers found that E. coli did not multiply and usually decreased during refrigerated storage. Enterococci usually remained practically unchanged for about 2 weeks, and often increased after 3 to 4 weeks of storage.

Gibbons (1934) and Griffiths (1937) reviewed the literature on the occurrence of E. coli and related lactose-fermenting organisms in fish. A number of workers found that E. coli was not a normal inhabitant of the intestinal tract of fish, but was present along with other organisms of the coli-aerogenes group in fish taken from polluted

waters (Fellers, 1926; Gibbons, 1934; Hunter, 1920; Johnson, 1904; and Zobell, 1941). The presence of coliform bacteria, including typical E. coli, in iced market fish was noted by Green (1920), and Griffiths and Fuller (1936).

Green (1949d) demonstrated that 49% of 41 samples of freshly caught non-iced shrimp contained members of the coli-aerogenes group of bacteria. The coli-aerogenes group was found more frequently in or on the "head" (cephalothorax) and in the slime on the head than in headless shrimp (abdomen and tail). All of 14 samples of market shrimp examined contained coliform bacteria; 57% contained E. coli. E. coli was found in 62% of the samples after two months frozen storage, and at this time all samples were positive for the coli-aerogenes group. Since E. coli is rarely encountered in freshly caught shrimp, the occurrence of this organism in appreciable numbers in market shrimp is indicative of the need for more rigid sanitation in the handling of this product.

Aquatic edible molluscs tonged from sea water near Alexandria, United Arab Republic were found to be grossly polluted when examined by Moussa (1964). Most of the samples examined showed exceptionally high counts of coli-aerogenes bacteria and enterococci.

In 1902, Fuller traced the sewage flow of Providence, Rhode Island, which discharged approximately 14 million gallons of sewage per day into Narragansett Bay. Water and oyster samples were

collected from various locations in the bay, and analyses showed that water, oysters, mussels, and clams from beds near the sewer opening contained E. coli and Aerobacter aerogenes. This study indicated that oyster beds can become contaminated at considerable distances from the source of pollution. Many European countries were confronted with health problems due to contaminated oysters during the latter part of the nineteenth century. Numerous papers and articles published during this period offered many comments in which typhoid fever cases were related to oysters which were contaminated with the causative agent.

Tobin and McCleskey (1941) observed that analyses of a large number of samples of fresh crabmeat revealed that a high per cent of samples were contaminated by E. coli. Total bacterial count, on standard plate count agar, varied from 87,000 to 16,000,000 per gram. In general, a high total count was associated with the presence of E. coli.

In recent years public health officials and food processors have been concerned with the increasing incidence of infection and resulting illnesses caused by the various serotypes of organisms belonging to the genus Salmonella. Salmonellosis is recognized to be predominantly a food-borne disease, and Salmonella sp. are harbored by both man and animals (Granville, 1963 and Little, 1965). Many food processors are establishing specifications for salmonella-free

purchases. Many countries have instituted regulations to control the conditions under which items subject to Salmonella sp. infection can be imported (Hansen, 1963; Little, 1964; Miller, 1963). As regulations become more stringent, and as the public becomes more aware of the problem, food producers and processors will find it necessary to safeguard their products from Salmonella sp. to maintain a continuing market. The organism responsible for salmonellosis can be destroyed by thorough cooking (Weiser, 1962). The greatest danger has involved uncooked foods or food prepared and contaminated by food handlers. Since many of the crayfish are processed for distribution and marketing as iced, fresh crayfish, salmonellosis caused by contaminated crayfish meat can be a public health problem of growing importance. The significance of salmonellosis may be judged from the burgeoning body of literature devoted to the subject (Bowner, 1964; Hardy et al., 1942; Olitzky, 1956; Slocum, 1963; and Williams, 1964). Furthermore, many U. S. and foreign governmental agencies have been established specifically to monitor and combat the disease. Notable in the U. S. in this respect is the Salmonella Surveillance Unit of the Communicable Disease Center, Atlanta, Georgia (Little and Caker, 1964).

The primary cause for concern regarding Salmonella sp., as a source of human illness, is the rapidly increasing number of cases reported each year. In the U. S. 18,649 cases of Salmonellosis

were reported for the year 1963 (Salmonella Surveillance, 1964). The number of fatalities from Salmonellosis was small, and those where death resulted were primarily among the aged or the very young.

Steele (1963) stated that the genus Salmonella came into prominence in the late nineteenth century as a result of work by Salmon and Smith, and first become an important problem to the food industry during World War II. The disease, Salmonellosis, may be caused by any one of many serotypes of Salmonella sp. that are known to exist.

Salmonella typhimurium is the type now most frequently associated with salmonoellosis in the U. S. (Bowner, 1965 and Williams 1964), and other types common to both man and animals are S. montevideo, S. infantis, and S. heidelberg. In fact, more than 800 different species of Salmonella organisms have been identified (Little, 1965).

Salmonellosis has been generally contracted by the ingestion of food contaminated with Salmonella sp. Feces of infected animals contained large numbers of these organisms. Salmonellosis is considered primarily an excremental disease, and transmission is by the fecal-oral route (Bowner, 1964).

The human element also played an important role in the contamination of foods. A food handler may be a carrier of S. typhimurium,

S. schottmeulleri, S. newport, S. enteritidis, and S. cholerae-suis (Weiser, 1962). Some persons have become infected with the organisms without exhibiting symptoms, or they have harbored the bacteria for long periods after they have recovered from the initial effects of the disease. These persons, particularly if they were handling food, continued to transmit the disease to others (Bowner, 1964; McCroan et al., 1963). Unsanitary practices in food processing plants readily permitted the growth and transmission of Salmonella sp. to populations producing public health hazards.

A wide variety of foods have been implicated as sources of Salmonella sp. Although this report deals principally with crayfish, a cursory investigation of the literature has provided additional information concerning its occurrence in other foods and food products.

Recent publications have indicated the absence of Salmonella sp. and other enterobacteria of fecal contamination in marine fish caught in open seas. However, observations of their presence in fish when marketed fresh or dispatched from filleting or icing establishments have been reported (Griffiths, 1937; Larkin et al., 1956; Olitsky et al., 1956; Shewan and Liston, 1955).

Very little information concerning incidence of Salmonella sp. in shellfish is available in the literature; however, the following investigations have been noted.

Investigations to determine the relative survival of S. schottmulleri in oysters stored under conditions simulating commercial practice were conducted by Kelly and Arcisz (1954b). Shellfish were allowed to feed in sea water containing added suspensions of the test organisms. After definite time intervals, survival rate was determined by quantitative methods. S. schottmulleri were recovered in significant numbers from oysters after dry storage at 40°F for over 50 days. This indicated that the organism could persist in oysters for a period of time comparable to that involved from harvest to consumption.

Incidence of survival of Salmonella and Shigella indicator organisms in shellfish grown under various conditions has been carried out by Kelly and Arcisz (1954b). As a result of this work, practical recommendations necessary for prevention and control of shellfish-borne infection were established.

Buttiaux (1962) found that Salmonella sp. survived in marine environments long enough to be ingested in a fully viable stage by oysters. Hence, being completely immobile, oysters cannot benefit from any self-cleansing of water; the waters in which they live can, in fact, be repeatedly polluted.

Finally, those enterobacteria that were absorbed survived for long periods after the catch. As early as 1928, it was shown that S. typhi remained viable in fresh oysters during transportation to the

consumer. Similar conclusions were derived from experiments with S. paratyphi in flesh of oysters (Kelly and Arcisz, 1954b). These workers also conducted investigations to determine relative survival of S. schottmulleri and E. coli in shell oysters and soft clams, stored under conditions simulating commercial practice. The rate of reduction of Salmonella was not as great as that of E. coli during usual storage periods. Reduction in numbers of both organisms ran more parallel in soft clams. However, both organisms persisted in shellfish for a period equal to that of harvesting to consumption. There was little evidence of multiplication of these organisms in shellfish still in marketable condition, except in oysters or clams that had died or had become so weakened that putrefaction had started at the time of harvesting.

Species of Shigella, causing bacillary dysenteries, may be transported in foods. Infections caused by dysentery bacilli are probably far more common than is generally recognized. In 1945, 33,495 cases and 400 deaths were reported from 38 states (Burrows, 1964). Dysenteric infections appeared most common in the summer months in hot countries with temperate climates, although they could occur at any season of the year. The spread of disease was due to more or less direct transfer of specific bacillus from infected intestinal discharges to the alimentary tract of a fresh individual. Polluted water played a part in some outbreaks, but it apparently

was not nearly as important a factor in dysentery as it was in typhoid fever.

A number of cultural and biochemical criteria of more resistant enterococci were used collectively, by Moussa (1965), to speciate 2,477 enterococci strains isolated from water, soil, vegetation, sewage and human and animal feces into 2 major groups: Streptococcus fecalis and S. fecium. S. fecalis was found to predominate in human and poultry gut, S. fecium predominated in livestock gut, while both organisms were about equally distributed in water, soil, vegetation and sewage samples.

S. fecalis and S. fecium have been found in human and animal feces in numbers as high as those of the coli-aerogenes organisms, S. fecalis however predominated in human and poultry guts (Barnes, 1959; Kjellander, 1960) while distribution of S. fecium showed a wider range. Many workers (e.g. Brown and Gibbons, 1950; Ingram and Barnes, 1955; Guthof and Dammann, 1958; Buttiaux, 1959) emphasized the importance of enterococci as indicators of fecal pollution. Enterococci were readily isolated in large numbers from human and animal feces, and the relative resistance of these bacteria to adverse conditions proved advantageous in bacteriological examination of sea water, soft drinks and dried, frozen and processed foods.

Consistently high recoveries of enterococci as compared to low numbers of coliforms obtained from the same samples of frozen sea

foods were indirect evidence that enterococci were better indicators of contamination in such foods (Raj et al., 1961). The use of azide dextrose broth, modified by incorporation of bromthymol blue, and of ethyl violet azide broth as presumptive and confirmation tests, respectively, were highly specific for detection and enumeration of enterococci in food samples. Tetrazolium agar medium, when used as a third step after the confirmation test, provide a reliable differentiation of S. fecalis types from other group D streptococci.

Larkin et al. (1956) made a comparison between MPN of coliform bacteria and fecal streptococci in frozen fish and fish products. The use of fecal streptococci as test organisms was advocated for bacteriological examination of frozen fish products. Testing procedures for isolation of fecal streptococci were simpler and results more significant than those of coliform bacteria,

The first recognized outbreak of botulism was observed over 200 years ago, although the causal organism was not isolated until 1895. The disease is caused exclusively by the ingestion of food in which C. botulinum has grown and produced its toxin. According to Lamanna (1959), botulinum toxin is the most potent poison known to man; less than 1×10^{-10} will kill a mouse.

Before 1963 the American public was hardly aware that type E toxin existed. In 1960, three women in Detroit, Michigan, ate a lunch of tuna fish salad and two of them died (Johnston, 1963).

C. botulinum, type E toxin was diagnosed. Resulting publicity served effectively to introduce C. botulinum, type E toxin to the general public.

Spores of C. botulinum are widely distributed in nature, having been found commonly in soil, mud, and intestinal contents of animals. Thus the opportunity for contaminating food existed almost everywhere.

Bott et al. (1966) reported that the intestinal contents of more than 3,000 fish from Lake Erie, Superior, Huron, and Michigan were examined for C. botulinum, type E toxin. Demonstration of the organism was accomplished by identifying its toxin in liquid cultures inoculated with material from the alimentary tract.

Meyer (1953) defined botulism as a specific intoxication with toxins of C. botulinum or parabotulism. Six serotypes, A, B, C, D, E, and F exist in nature as sporulating saprophytes and grow freely in a great variety of inadequately preserved animal or plant foods. During resultant spoilage a powerful toxin was formed and on ingestion was absorbed, ultimately inducing some changes in motor-nerve terminals at the neuromuscular junction: acetylcholine output was diminished, with effects resembling denervation.

C. botulinum, type E toxin, one of the causative agents of botulism outbreaks, has a worldwide distribution. Kamizawa (1960) and Kakamura et al. (1956) have demonstrated its presence repeatedly in soil and mud samples on Hokkaido. Johannsen (1962, 1963) has

isolated the organism from large numbers of soil, seashore, and sea bottom samples in and near Sweden. Pederson (1955) has found it in soil and bottom mud in Denmark. Dolman (1963) has isolated the organism repeatedly from bottom samples off the coast of British Columbia. A review of Public Health Records of the Gulf States did not reveal any cases of type E botulism attributed directly to Gulf States shrimp and oysters. Previous preliminary word on shellfish by Novak (1961) revealed that he found no C. botulinum type E in shrimp or oysters.

According to Grodner (1964), all tests were negative for C. botulinum and type E toxin in Gulf shrimp, caught and processed through normal channels in processing and packing plants were negative.

Over 700 pounds of fresh Gulf shrimp from Texas, Louisiana, Mississippi, Alabama, Georgia and Florida, headless and whole, including the intestinal tract, commercial frozen peeled and deveined, and breaded, were examined for presence of C. botulinum and type E toxin (Grodner, 1965). No evidence of C. botulinum and type E toxin was found. He also reported an absence of C. botulinum and type E toxin in fresh Gulf oysters and frozen breaded oysters from Louisiana, Alabama, Georgia and Florida.

Grodner (1966a and 1966b) examined samples from over 1200 pounds of fresh Gulf shrimp and over 300 pounds of fresh Gulf oysters from the Gulf Coast for presence of C. botulinum and type E toxin

with similar results. Samples were tested for other pathogens with potential health hazards from Salmonella and coliform groups by standard methods.

In an investigation of several Japanese baby clam canneries, Amano (1948) found great tolerant microorganisms of the genera Bacillus and Clostridium in soils, rubbish, factory wastes, sand, and sea waters surrounding processing plants. No C. botulinum or any related toxin was present. Apparently, anaerobic bacteria are the principal viable organisms in shells of living baby clams. A heat-resistant anaerobe related to C. bifermentans was isolated from fresh clam meat of the species Venerrupis philippinarum.

According to Eklund and Poysky (1965) C. botulinum type F toxin has been demonstrated in two samples of marine sediments. One sample was taken 83 kilometers off the coast of California; the other, 100 kilometers off the coast of Oregon. Cultures of this type have not been reported previously in the U. S., and only once before in the whole world.

Meisel (1964) isolated a strain of C. botulinum type E toxin that caused food poisoning in man, in Poland, from canned fish. This strain was highly saccharolytic but weakly proteolytic. The toxin was rather weak, and the prototoxin strongly activated by trypsin. Immunologically, the toxin was idential with toxins from type E strains isolated in France and the U. S. S. R.

The majority of the samples of C. botulinum, type E toxin have been isolated from marine fish and the marine environment; very few have been from freshwater lakes and streams. Chapman and Naylor (1966) conducted a study of the isolation of C. botulinum, type E toxin from fish taken from Lake Cayuga, a freshwater lake in New York State.

Nickerson and Goldblith (1964) reported there had been no outgrowth with and toxin production in any sample of shucked clams inoculated with C. botulinum type E, at 10^2, 10^4, and 10^6 spores per gram of Alaska, Beluga, Minneapolis, and 8E strains after irradiation at 350, 600 and 830 Krads stored at 35°F or lower. These workers further showed that shucked clams inoculated with 10^2, 10^4, and 10^6 mixed spores per gram of the same 4 strains of C. botulinum irradiated at 350 Krad produced toxin after 27 days at 40°F. Shucked soft-shelled clams inoculated with 10^4 and 10^6 mixed spores per gram of the same 4 strains of type E, irradiated at 600 Krad produced toxin after 90 and 47 days, respectively at 40°F.

Many beta-hemolytic coagulase-positive strains of Staphylococcus aureus are pathogenic, and some produced an enterotoxin causing food poisoning. Appreciable levels of enterotoxin were produced only after considerable growth of the organism; usually a population of at least several millions per milliliter or gram must be attained. It has been observed that production of

enterotoxin by staphylococci was more likely when competing microorganisms are absent, few, or inhibited for some reason. Therefore, a food that had been contaminated with staphylococci after a heat process would be favorable for toxin production. There are no reliable figures on numbers of cases of staphylococcus poisoning in the U. S. or in any states for any given period. Poisoning usually is not reported or publicized unless the outbreak is fairly large, as at a picnic, large dinner, or convention. It is known, however, that a large percentage of all cases reported as "food poisoning" or food infection actually are staphylococcus poisoning, and that most of us encounter this illness a number of times during our lifetimes. Of the many kinds of food that have been involved in causing staphylococcus food poisoning, custard-filled and cream-filled bakery goods, ham, tongue, and poultry have caused the most outbreaks. Other foods incriminated include meats and meat products, fish and fish products, milk and milk products, cream sauces, salads, puddings, custards, pies, and salad dressings.

Most attention has been paid to the presence of S. aureus in various fish and fishery products and to the usefulness of various media for this organism's detection and enumeration. It perhaps should be pointed out that selective media depend on certain properties of S. aureus, e.g. presence of phosphatase, fermentation of mannitol, tolerance of tellurite or 10% NaCl, or the clearing of an opaque

egg yolk agar plate. However, it is possible that some entero-toxigenic strains may not possess one or the other of these properties and hence could be missed by the use of only one of the selective media. Although most virulent strains are coagulase positive, coagulase negative strains cannot be ignored (Hopton, 1961).

Cultures of *E. coli*, *Proteus* sp., *S. aertrycke*, *S. dysenteriae* (Flexner) and *S. aureus* were inoculated, respectively, into autoclaved crabmeat and the rate of growth observed (Berry, 1942). Significant increases in bacterial plate counts were observed with each culture employed when the incubation temperature was 25°C, however, bacterial numbers decreased, although viable organisms were still present in the crabmeat after 15 days at this temperature.

Sinskey et al. (1960) demonstrated that certain organisms of public health significance and other contaminants in model systems and foods can survive freeze drying to various extents, and that survival was influenced by the composition of food to be freeze dried and by the processing temperature. Saleh et al. (reviewed by Pablo et al., 1966) surveyed eight randomly selected commercial freeze dried foods and detected organisms of public health significance. Microbial flora of freeze dried food was related to, but not necessarily identical to that of the original raw material. Therefore, both for product quality and for anticipating specific situations of potential

public health significance, it was important to predict growth patterns of various groups of organisms subsequent to rehydration at specific temperatures. Microbial growth patterns of rehydrated shrimp were investigated at 4, 20, and 37°C. Storage temperatures greatly influenced growth rates. The lag period was longest at 4°C and shortest at 37°C. The maximum population was reached twice as fast at 37°C as at 20°C. Samples stored at 4°C increased 10,000-fold in total aerobic counts but required two weeks to do so. Growth patterns indicated a shift in microbial spectrum in response to temperature. This was pronounced when rehydrated shrimp were stored at 4°C at which the mesophilic population became a minority. It was apparent that, in common with other types of perishable food products, the storage life of rehydrated shrimp can be significantly extended by low temperatures. The shrimp storage life was 7 times as long at 4°C as at 20°C, and 20 times as long as at 37°C.

Bacterial counts on fresh shrimp in all stages from the time caught, through heading, washing, and icing until delivery in consumer markets, have been recorded by Green (1949a). Fourteen representative samples of freshly caught, whole Gulf shrimp averaged 42,000 bacteria per gram. The majority of these bacteria were present in the surface slime and in the "head" of shrimp. Heading and thorough washing reduced the bacterial count per gram by approximately 75% and 40%, respectively. Thirteen samples of freshly

caught, headless, washed shrimp contained an average of 7,400 bacteria per gram.

The bacterial count during 7 to 10 days' iced storage in the bottom layer of the bin increased steadily to a million, or more per gram. Shrimp packed in upper layers of an ice bin had much lower counts than those of similar age in the bottom layers, the average count on two composited samples from one trawler, when unloaded, was 55,000 per gram. Spoiled shrimp varied widely in total numbers of bacteria, 12 samples of headless spoiled shrimp averaged 288,000,000 bacteria.

Refrigeration without direct contact with ice produced a superior product. The bacterial count after 7 days' storage approximated the freshly caught product and indicated that use of mechanical refrigeration should be investigated further. Twenty-six samples of market shrimp, purchased from wholesale and retail dealers, averaged 32,600,000 bacteria per gram. A comparison of this figure with those on freshly caught and iced shrimp indicated that better refrigeration methods are needed in transit to market, as well as on fishing boats.

Green (1949c) recorded bacterial counts on frozen shrimp of known pre-freezing history and on frozen shrimp from various commercial freezing plants after varying periods of storage at -17.8°C. After 12 months' frozen storage, shrimp on board the trawler immediately after catching, heading, and washing, contained 22,000 bacteria

per gram. Headless shrimp held 9 days in iced storage contained more than a million bacteria per gram after the same storage period. Lowest counts were found on shrimp which had been refrigerated without direct contact with ice before freezing. An average reduction in bacterial count of 50% was recorded on market shrimp after one days' frozen storage; this was increased to 82% reduction by two months' storage. Percentage reductions produced by from 2 to 12 months' frozen storage of laboratory and commercially frozen products ranged from 48 to 99.2%. Bacterial counts on commercially frozen and stored shrimp varied widely and showed only a slight correlation with organoleptic examination.

Crabs are characterized by particularly rapid and deleterious changes in their meat, starting at death at which time they readily succumb to microbial attack. However, the meat of newly caught crab has been referred to as almost sterile, but bacteria multiplied rapidly (Timofeev, 1949a). The bacterial invasion started primarily from the intestinal tube.

The bacteria appeared to be mainly on the surface of the body; relatively few were found in the intestinal tract (Goresline and Smart, 1942). A study of crabs and crab mean by Harris (1932) led to the conclusion that decomposition was due principally to organisms of the Proteus, Pseudomonas, and Flavobacillus groups. E. coli was present at 20°C during early stages of storage but almost absent when

kept at 2 to 5°C. In another study, organisms belonging to the Escherichia, Sarcina, and Streptococcus genera were isolated. It was concluded that decomposition was due to progressive proteolysis by organisms belonging to the Proteus group. This was confirmed by Soviet investigations (Timofeev, 1949a), who claimed that bacteria of this latter group multiply rapidly in crabs, but signs of decomposition were slow to appear. Food poisonings through crabs have been attributed to proteus attacks (Timofeev, 1949b).

Goresline and Smart (1942) studied the blue crab (Callinectes sapidus) in its soft-shell stage. Bacterial counts from several lots of fresh crabs were fairly uniform. Bacteria were mainly on the surface of the body and relatively few were found in the intestinal tract or body fat, even in crabs which had been dead for a number of hours. Blanching in hot water lowered the bacterial count, but caused crabs to be rather soggy when fried and did not enhance the keeping quality. E. coli were found on a few fresh crabs but not on those in storage in the frozen state. There was no clearcut correlation between the quality of crabs before freezing and bacterial content of the product after storage in the frozen state.

The number of reports on bacteriology of precooked frozen seafoods published in the last 20 years is quite scarce. Proctor and Phillips (1947, 1948), in reporting a study on a number of frozen foods, stated that 85% of precooked fishery products examined showed

plate counts in excess of 10^4 per gram. Gunderson et al. (1954) examined frozen raw breaded shrimp and found coagulase-positive staphylococci in all samples, viable counts of 10^5 to 10^7 per gram, coliform counts of 10^2 to 10^4 per gram, but no Salmonella or Shigella. Larkin et al. (1956) examined precooked frozen fish sticks and reported viable counts of 10^3 per gram, enterococci of 10^2 per gram, and coliforms of 10^1 per gram. These workers suggested that the breading procedure was most likely to be a major source of contamination.

Kern (1957) made bacterial studies of shrimp and found all but one of the samples of unfrozen raw shrimp and of frozen green shrimp had total counts over 10^5 per gram. Silverman et al. (1961) examined frozen raw and cooked shrimp and found that although most of the uncooked samples had counts of 10^5 to 10^6 per gram, most cooked samples showed counts of 10^3 per gram or less. Uncooked samples generally contained coagulase-positive staphylococci, but these were absent from most cooked samples.

Thermal destruction rates of S. facalis, ATCC 7080, were determined by Ott, et al. (1960) in a selected group of meat and fish precooked frozen products. It was concluded that the procedure adopted for establishing thermal treatment was accurate and proved feasible for this type of product. Based on thermal resistance of this test strain, heat treatments recommended on commercial packages seem

to be adequate, provided the heating and cooling procedures were similar to those followed in these experiments. In the case of one product out of five tested, however, the minimum recommended heating period fell short of the computed thermal requirement for that product.

Raj and Liston (1961a), while working with frozen processed foods stored at 0°F for periods up to 648 days, demonstrated that total viable counts at 68 and 95°F showed less than a ten-fold decrease. Coliform counts declined comparably in uncooked samples but more steeply in precooked samples, and enterococci showed fluctuations but little or no net decrease in either type of sample. Studies of the effects of freezing (-30°F) and storage at 0°F on six selected species of microorganisms associated directly or indirectly with potential food-poisoning hazards showed that in brain heart infusion broth suspensions, after 393 days at 0°F, S. typhimurium was virtually eliminated, E. coli and S. aureus were greatly reduced in number, S. pyogenes counts were slightly reduced, and S. bovis and C. perfringens were little affected. When suspended in sterilized fish homogenate, S. typhimurium and S. aureus were reduced ten-fold in 393 days at 0°F, while the rest of the cultures were not significantly affected.

It was quite obvious that fish and fish products could be the source of food infection and poisoning (Shewan, 1961, 1962; Guelin, 1962; Buttiaux, 1962; Georgala, 1957; Abrahamson, et al., 1959;

Silverman, et al., 1961 and Dussault, 1962). Appleman, et al. (1964) have suggested that the incidence of the common food poisoning organisms in such foodstuffs was quite low and that fish, in this country at least, was one of the safest articles of diet.

Raj and Liston (1963) made a bacteriological study of a processed frozen seafood plant, its products, and comparable products obtained from retail stores. A total of 362 samples were examined. Each sample was simultaneously tested for total count at 20 and 35°C, most probable numbers of coliforms, enterococci, hemolytic streptococci, E. coli, and coagulase-positive staphylococci, and the presence of Salmonella, Shigella, and sporeforming anaerobes. Frozen raw seafoods entering the plant carried comparatively low levels of bacteria of public health significance. The initial cutting operation caused a ten-fold increase in counts and introduced significant contamination by coliforms and enterococci and coagulase-positive staphylococci. Breading and bettering operations increased the contamination of the product with coliforms, enterococci hemolytic streptococci, anaerobes, and coagulase-positive staphylococci. The precook operation reduced total counts, coliforms, and hemolytic streptococci, and anaerobes. Enterococci could be used as a better index of fecal contamination than coliforms in these samples. Retail samples of uncooked and precooked frozen seafood products yielded bacteriological results similar to those obtained from plant samples.

Pasteurization of crab meat was undertaken, by Anzulovic and Reedy (1942a), to extend keeping quality for extended shipments and to destroy any pathogenic organisms that could contaminate crab meat during handling. Crab meat from each can was divided into two portions, packed, sealed, and pasteurized at one of the following temperature ranges; 145°F for 30 min; 150°F for 20 min; 155°F for 15 min; 160°F for 10 min; and 170°F for 1 min. Pasteurized lots of crab meat were opened at weekly intervals to find out the length of time crab meat would keep in the refrigerator at 41°F to 43°F. These were compared with freshly purchased crab meat in regard to color, odor, and taste. It was found that pasteurized crab meat kept as long as 5 weeks at 41 to 43°F, was free from E. coli, contained a small number of bacteria, and the color, odor, and taste were not impaired.

MATERIALS AND METHODS

I. Incidence of Microorganisms of Public Health Significance

A. Collection of Whole Live Crayfish for Microbiological Analysis

Crayfish samples used for the survey of public health significant organisms were collected from their natural habitat with chicken wire crayfish cages or set nets normally used by the Louisiana commercial crayfishermen. Individual crayfish were taken from these traps by sterile forceps and placed into sterile 32 ounce specimen bottles and immediately capped loosely to prevent suffocation of the crayfish. All sample bottles were packed in ice and returned to the laboratory for examination within a few hours. On occasions, when this method of obtaining crayfish was inconvenient, they were picked, aseptically, from the center of the fisherman's sack soon after reaching the banks or processing points.

B. Locations and Collection Schedule of Whole Live Crayfish

The following areas were sampled once every 3 to 4 weeks. Collections began on February 15, 1966 and extended through June 7, 1966.

1. Atchafalaya floodway in the Henderson area
2. Atchafalaya floodway, Grand River flats

3. Woodland pond, Wade O. Martin
4. Ricefield, L. Miller
5. Swamps, near Lake Verret
6. Swamps, near Pierre Pass
7. Atchafalaya floodway, Opelousas Bay
8. Marsh, near Pecan Island
9. Woodland pond, Elmer Naquin
10. Ricefields, near Henderson
11. Atchafalaya floodway, near Pierre Pass
12. Swamp, Grand Bayou
13. Ricefield, John Ruppert
14. Ricefield, Roland Faulk
15. Atchafalaya floodway, near Rosedale
16. Atchafalaya floodway, from Henderson
17. Marsh, near Forked Island
18. Atchafalaya floodway, Bayou Pidgeon
19. Open land (pasture), near Pecan Island
20. Atchafalaya floodway, Belle River
21. Atchafalaya floodway, Breaux Bridge
22. Natural Lake, Lake Pearl

C. Collection of Fresh Water Samples for Microbiological Analysis

Water samples were collected in sterile 32 ounce screw cap bottles. Several samples were taken from different areas of each

location. Water samples were taken to the laboratory for microbiological analysis as soon as possible.

D. Locations and Collection Schedule of Fresh Water Samples

The following areas were sampled every 3 to 4 weeks. Collections began on February 15, 1966 and extended through June 7, 1966.

1. Atchafalaya floodway in the Henderson area
3. Woodland pond, Wade O. Martin
5. Swamps, near Lake Verret
6. Swamps, near Pierre Pass
7. Atchafalay floodway, Opelousas Bay
8. Marsh, near Pecan Island
9. Woodland Pond, Elmer Naquin
11. Atchafalaya floodway, near Pierre Pass
13. Ricefields, John Ruppert
15. Atchafalaya floodway, near Rosedale
16. Atchafalaya floodway, from Henderson
17. Marsh, near Forked Island
18. Atchafalaya floodway, Bayou Pidgeon
20. Atchafalaya floodway, Belle River
21. Atchafalaya floodway, Breaux Bridge

E. Preparation of Samples for Examination

Five crayfish weighing approximately 20 gram each were placed into five separate sterile screw cap Waring Blendor jars. Each sample was diluted 1:10 with Butterfields Phosphate Buffer (Appendix E) and blended for two minutes. This mixture was then used in analyses for selected pathogenic organisms.

F. Coliform Analysis

A 3-tube most probable number (MPN) series was inoculated with dilutions of 1:10, 1:100, and 1:1,000 (i.e. 3 tubes per dilution) using lauryl sulfate broth (LST, Difco, 1966) and incubated at 37°C for 48 hr. LST fermentation tubes were examined for gas formation after 24 and 48 hr. From each gas positive tube, one loopful was transferred to 2% brilliant green lactose broth (BGL, Difco, 1966). BGL broth fermentation tubes were incubated at 37°C for 48 hr. MPN were computed on basis of the gas positive BCL broth tubes and reported as coliforms per gram (Fishbein, 1962).

G. Fecal E. Coli Analysis

Fishbein (1962) described a method by which E. coli group and strains of Aerobacter could be separated by use of EC broth (Difco, 1966) and elevated incubation temperatures. In EC broth it was found that as a group atypical E. coli were the most resistant to gas-positive types. Lease resistant in EC broth was a group of known typical fecal

isolates of E. coli. Of intermediate resistance between the two groups was the large body of typical E. coli. The broth from positive tubes of LST were transferred by loop to EC broth. These tubes were incubated for 48 hr at 45.5°C ($\pm$0.1°C) in a water bath. Gas-positive tubes were streaked on Eosin Methylene Blue (EMB) agar (Difco, 1966) and incubated for 24 hr at 37°C. Typical E. coli colonies were verified by indole, methyl red, Voges-Proskauer, and citrate (IMViC) reactions of ++--. Throughout this paper, all E. coli groups which were grown in EC broth at 45.5°C and gave an IMViC reaction of ++-- will be referred to as fecal E. coli. This is the group which Fishbein regarded as being least resistant in EC broth at 45.5°C. The MPN was computed on the basis of gas positive EC broth at 45.5°C with an IMViC pattern of ++-- and was reported as fecal E. coli per gram.

H. Coagulase-positive Staphylococci Analysis

Serial dilutions of the blended mixture were planted in trypticase soy broth (Difco, 1966) with 10% NaCl. One tube of the medium was inoculated with each of five dilutions (10^{-1} to 10^{-5}). Tubes were incubated at 37°C for 48 hr. Each tube showing evidence of growth was streaked on Mannitol egg agar base (MEA) (Difco, 1966). Colonies showing a zone of precipitate after 24 hr of incubation were regarded as possible coagulase-positive staphylococci. These colonies were confirmed by use of Bacto-Coagulase Plasma (Difco, 1966). Results

were reported in dilutions in which coagulase-positive staphylococci were present.

I. Salmonella-Shigella Analysis

Salmonella and Shigella sp. were determined by inoculating 10 ml amounts of the blended mixture into 100 ml of selenite cystine broth (Difco, 1966) and 100 ml of tetrathionate broth (Difco, 1966) and were allowed to incubate at 37°C for 24 hr. At this time plates of Salmonella-Shigella agar (SS) (Difco, 1966), bismuth sulfite agar (Difco, 1966) and brilliant green agar (Difco, 1966) were streaked with each of the two enrichment broths. These plates were incubated at 37°C for 48 hr. Colorless colonies from SS agar, black colonies from bismuth sulfite agar, and pink colonies from billiant green agar were transferred separately into triple sugar iron (TSI) agar slants (Difco, 1966) and incubated at 37°C for 48 hr. All slants showing alkaline slants and acid butts were transferred to Urease medium (Difco, 1966) to screen out Proteus sp. Growth from TSI slants showing negative for gas and negative for H_2S were biochemically examined for Shigella sp. All others were examined biochemically and serologically for Salmonella sp. Results were reported as positive or negative.

J. Fecal Streptococci Analysis

A one milliliter portion from each selected dilution of samples were transferred into 3 tubes of azide dextrose broth (Difco, 1966).

All tubes were incubated at 37°C and observed for growth after 24 and 48 hr. Each tube showing growth was considered as presumptively positive. A loopful of broth from each positive tube in the last 3 positive dilutions was transferred to a tube of ethyl violet azide broth (Difco, 1966). Tubes were incubated at 37°C and observed for turbidity after 24 and 48 hr. MPN of fecal streptococci per gram of sample was reported.

K. Clostridium Botulinum Type E Toxin Analysis

Each of 2 tubes of freshly boiled and cooled trypticase, glucose, peptone (TGP) medium (Appendix B) were inoculated with 5 ml of the 1:10 mixture. Tubes were incubated at 30°C for a minimum of 3 days and examined periodically for gas production and growth. To a 1:2 ml aliquot from this culture, especially the sediment, an equal amount of absolute ethyl alcohol was added in a sterile test tube. The mixture was allowed to stand at room temperature for a minimum of 1 hr, with occasional mixing. Following the 1 hr treatment, plates of liver veal agar containing 4% egg yolk (LVEY) (Appendix A) were streaked with a large loopful of the mixture. Plates were incubated anaerobically (10% CO_2 and 90% N_2) for 48 hr at 30°C. To expedite recovery of toxigenic cultures, tubes of TGP broth were also inoculated with 0.1 to 0.2 ml of the alcohol treated suspension at this time. After 48 hr plates were examined for typical C. botulinum, type E toxin colonies. On LVEY type E colonies appear as white irregular colonies, 1 to 2

mm in diameter, surrounded by a zone of yellow precipitate approximately 5 mm in diameter. On standing, clear zones usually appeared around a zone of precipitation. These characteristics are not specific but rather general for some spore-formers; so caution was necessary in the interpretation of results. Where typical C. botulinum, type E toxin, colonies were noted in considerable numbers with little or no contamination present, the corresponding TGP broth tube may be tested for type E toxin after 3 days' incubation at 30°C. Results were reported as presence or absence of C. botulinum, type E toxin.

II. Storage Growth Studies of Microorganisms in Crayfish Products

A. Organisms Used to Study Growth Patterns in Raw and Cooked Crayfish Meat

1. Salmonella typhimurium (Loeffler)

   American Type Culture Collection Number 13311.

2. Staphylococcus aureus

   American Type Culture Collection Number 9664.

3. Shigella dysenteriae strain 12

   Mr. A. Abrams, Walter Reed Army Medical Laboratory, Washington, D. C.

4. Escherichia coli 0127 B:8

   Mr. A. Abrams, Walter Reed Army Medical Laboratory, Washington, D. C.

5. Streptococcus faecalis

American Type Culture Collection Number 7080.

B. Preparation of Inoculum

Each organism was grown on Brain Heart Infusion (BHI) slants (Difco, 1966) at 37°C for 24 hr. A Bausch & Lomb (Spectronic 20) Spectrophotometer set at a wave length of 600 millimicrons, after a few minutes warm-up, was adjusted to a zero reading. A sterile cuvette containing approximately 5 ml sterile Butterfield's phosphate buffer was used to adjust the instrument to a 100% transmittance. Then a cell suspension of the desired organism in sterile Butterfield's phosphate buffer was inserted in place of the standard and the percent transmittance of absorbance value read directly. BHI slants were washed with 1 ml of sterile buffer. This cell suspension was transferred, drop by drop, with a sterile pipette to a sterile cuvette containing 5 ml of buffer. The cuvette was inserted and the percent transmittance read. Readings were taken until 50% transmittance was obtained. The desired inoculum was determined by diluting this suspension and running total plate counts on them.

C. Preparation of Raw Crayfish Meat

Fresh live crayfish were washed and dipped into boiling water for 10 sec to kill them and facilitate peeling. The tail meat was removed from its shell, deveined, and washed with running tap water.

The clean "raw," meat was then packed in sterile containers and frozen at -20°C until needed. At the time of inoculation tails were thawed and 20 g were weighed into each sterile 6 oz Nalgene screw cap jar. This preparation was then ready to be inoculated with the test organism.

D. Preparation of Cooked Crayfish Meat

The crayfish meat used to study growth patterns of these organisms in cooked crayfish flesh was obtained from a commercial crayfish processing plant. The crayfish had been scalded for 5 to 8 min in boiling water, peeled, and deveined but not washed free of the adhering "fat." Twenty grams of meat were weighed into each 6 oz Nalgene screw cap jar and autoclaved at 15 lbs pressure for 15 min prior to inoculation with the test organisms.

E. Inoculation Technique for Growth Studies

Samples were inoculated with 1 ml of the desired cell concentration by using a 1 ml pipette. One pipette was used to inoculate all samples which were inoculated with the same organism. Containers were then closed tightly, shaken, and incubated at desired temperatures and times. Samples were agitated frequently during their incubation period.

F. Incubation Conditions During Growth Studies

Three temperatures were used for these studies; 0 (packed in ice), 5, and 25°C. For each temperature, two sets of samples were prepared for an analysis after 7, 14, and 21 days of incubation. One set of samples were also prepared for an analysis at zero time.

G. Control Samples

All controls were prepared in the same manner as test samples with no inoculum. Controls were run with each temperature and time change respectively.

H. Enumeration of Test Organisms in Mixed Cultures of Raw Crayfish Meat

The method employed for quantitative determination of fecal E. coli was the same as that which was described earlier under part I section "H" of Materials and Methods.

The technique used for enumeration of coagulase-positive staphylococci was described by Zebovitz, et al (1955) using tellurite-glycine (TG) agar. The medium was autoclaved at 15 lbs pressure for 15 min, cooled to approximately 50°C, tellurite added aseptically, and poured into sterile petri dishes (20 ml per dish). The surface of the solidified agar was dried by overnight incubation in inverted position. The surface plating technique of Snyder (1947) was employed in which 0.1 ml aliquots were spread uniformly over the surface of the

agar by means of a sterile glass rod bent in the shape of a hockey stick. Graduated 0.2 ml pipettes were employed to measure the 0.1 ml volumes. After inoculation, plates were incubated in an inverted position at 37°C for 24 hr. Coagulase-positive staphylococci produce black colonies. Further incubation up to 48 hr or longer may allow coagulase-negative staphylococci to grow and produce similar black colonies. It is advisable to prepare the basal medium fresh before each use. If the prepared basal medium is stored in flasks and bottles and reheated to melt the agar, growth of coagulase-positive staphylococci may be inhibited. However, once the TG agar has been poured into petri dishes these may be stored for as long as 30 days in a sealed container in the refrigerator without loss of selectivity. One per cent aqueous stock solution of tellurite may be sterilized by autoclaving at 15 lbs pressure for 15 min.

Enumeration of fecal Streptococci was described under part I section "K" in Materials and Methods.

Enumeration of Salmonella sp. by MPN technique was employed as prescribed by the American Public Health Association (1958). From the 1:10 mixture of blended material, 10 ml were pipetted into 3 replicate tubes containing 10 ml of double-strength selenite broth (Difco, 1966) for each serial dilution and shaken thoroughly. This gave an equivalent of 1.0, 0.1, 0.01, 0.001, and 0.0001 gram of crayfish per tube, respectively. Tubes were incubated for 24 hr at 37°C. At this

time a large loopful of broth was streaked on the surface of freshly prepared bismuth sulfite agar and SS agar plates. SS agar plates were incubated for 24 hr at 37°C and bismuth sulfite agar plates for 38 hr. Both were observed for colonies with Salmonella characteristics. These colonies were picked from each plate and inoculated into sulfide-indole-motility medium (SIM) (Difco, 1966) and in addition TSI agar slants were streaked and stabbed. All tubes were incubated for 24 hr at 37°C and observed for typical Salmonella reactions. The number of tubes considered positive for Salmonella in each dilution was recorded. MPN of Salmonella was computed and reported as Salmonella per gram of sample. These results were confirmed serologically with Bacto-Salmonella O antisera (Difco, 1966).

The above method was also used for the enumeration of Shigella sp. with slight modifications. All SIM and TSI tubes were observed for the presence of typical Shigella reactions. These results were determined by biochemical methods and the use of Bacto-Shigella Antisera set.

I. Total Aerobic Plate Counts on Crayfish Products

Standard plate count medium (Baltimore Biological Laboratory, 1966) was used for all work involving total aerobic plate counts. This medium is a medium of standard composition for use of enumerating bacteria in eggs and egg products, milk and other dairy products and materials of sanitary importance by the plate method. After appropriate

dilutions had been placed in Petri plates, abour 15 ml of melted agar cooled to 43 to 45°C was added to each plate. It is recommended that all plates be poured within 15 min of making the first dilution. After the agar has solidified, plates were inverted and incubated at 37°C. After the incubation period all colonies on plates which show between 30 and 300 colonies were counted and dilutions used recorded. To compute numbers of organisms per gram of sample, multiply total colonies per plate by the reciprocal of the dilution used.

III. Growth Curve Studies of Microorganisms of Public Health Significance in Crayfish Products

All materials and methods used for these determinations are the same as those used in Part II of Materials and Methods. The only variations were different storage times and temperatures. Storage temperatures were 5, 25, and 37°C. Storage times were 0, 3, 6, 9, 12, 18, 24, 30, 36, 48, 72, 96, 144, and 175 hr, respectively.

Crayfish etouffé which was cooked and frozen was used for the study of the growth characteristics in a prepared crayfish product. The sample was prepared by weighing 20 gram amounts of etouffé into each sterile 6 oz Nalgene screw cap jar. These preparations were then ready to be inoculated with each of the test organisms.

IV. Study of Toxin Production of Clostridium botulinum, Type E Toxin in Crayfish Products

A. Preparation of Spore Suspension

A spore stock culture of C. botulinum, type E toxin strain 8E was used in this experiment. This strain was provided by Dr. C. F. Schmidt of the Continental Can Company, Chicago, Illinois. The C. botulinum spore suspension was prepared according to Schmidt et al. (1962). Stock cultures of this organism were inoculated into 30 ml of trypticase peptone glucose (TGP) sporulating medium in 25 x 150 mm screw cap tubes and the suspension was heat-shocked at 80°C for 10 min. This suspension was then incubated at 30°C for 24 hr and 10 ml quantities were inoculated into 8 oz screw cap prescription bottles containing 200 ml of TGP medium and incubated at 30°C for 5 days. Spores were then harvested by centrifugation at 5,000 rpm and washed twice with 0.85% sterile NaCl solution. Spores were resuspended in sterile 0.85% NaCl solution and held under refrigerated storage at 5°C.

B. Preparation of Samples for Spore Study

Samples of raw and sterile crayfish flesh and a sterile precooked crayfish product were prepared by weighing 50 gram of each into separate blendors. The spore suspension was added along with 50 ml of Butterfields phosphate buffer. An inoculum of C. botulinum type E

strain 8E spores was added to each of 50 gram of raw crawfish tails, sterilized crayfish tails, and crayfish etouffé. The inoculum of 10^3 spores per gram of each sample was determined by anaerobic plate counts of TGP agar (Appendix C) at 30°C for 5 days. This mixture was blended for 1 min and transferred to sterile 32 oz Nalgene screw cap bottles to be stored at 0, 5 and 30°C under anaerobic conditions. Case Anaerobe jars size No. 2, obtained from Case Laboratories, Inc., were used with an anaerobic indicator. A 15 mm vacuum was pulled by use of a water aspirator and a mixture of 10% CO_2 and a N_2 balance was then injected into the jar. This was performed 4 times and the jars were incubated at their respective storage temperatures.

C. Indicator Solution for Anaerobiosis

Because methylene blue is capable of being colored in the oxidized state while colorless in the reduced, it was used as an indicator for the presence of oxygen in the anaerobic jars. When the indicator of anaerobiosis was needed, a test tube of 2 ml of it was heated in a cup of water until the blue color disappeared. This method was described by Fildes (1932) in which 3 stock solutions are used to make the indicator.

1. Six ml N/10 NaOH diluted to 100 ml with distilled water.
2. Three ml of 0.5% aqueous methylene blue diluted to 100 ml with distilled water.

3. Six grams glucose dissolved in 100 ml distilled water to which has been added a crystal of thymol.

Equal portions of the 3 solutions were mixed and used as the indicator.

D. Botulinum Type E Toxin Assay Technique

Samples were tested for toxin production according to the following schedule: 30°C - 0, 24, 48, 72, 96, and 120 hr; 5°C - 0, 7, 14, 21, 24, 27, 30, 33, and 36 days; 0°C - 0, 7, 14, 21, 35, 42, 49, and 56 days. A modification of the toxin assay procedure of Duff et al. (1956) was used, where 5 ml of sterile 0.85% NaCl solution was added to a 5 gram sample of inoculated crayfish meat and homogenized for 2 min in a sterile screw cap Waring Blendor giving a 1:1 dilution. This suspension was then centrifuged at 5,000 rpm for 10 min to separate the insoluble fragments. The supernatant fluid was removed and adjusted to pH 6.2 using 1 N NaOH or 1 N HCl.

Two ml of this solution were transferred to each of two 16 x 125 mm screw cap tubes. To one tube was added 0.2 ml of a 10% solution of trypsin (Difco, 1:250 activity) resulting in a 1% solution of trypsin. Trypsin digestion was carried out for 45 min at 37°C. Trypsin (0.2 ml) was added to the second tube and heated for 11 min at 100°C. A 1:10 dilution of trypsinized mixture of tubes 1 and 2 were prepared by adding 8 ml of gel-phosphate buffer, pH 6.2.

Two Carworth mice were each injected with 0.5 ml of type E antitoxin (5 international units). White male mice used throughout the

experiment were obtained from Carworth, Inc., New City, Rockland County, New York. Antitoxin was obtained from the Biological Reagents Section, Communicable Disease Center, Atlanta, Georgia. After 30 min 0.5 ml of the nonheated trypsinized 1:10 dilution was injected with 0.5 ml of nonheated trypsinized 1:10 extract followed by a second pair of mice injected with 0.5 ml of the 1:10 heated trypsinized extract. Inoculated mice were maintained for 48 hr and observed at frequent intervals. Type E toxin was considered to be present in samples only if 1) mice protected with type E antitoxin survived; and, 2) the mice injected with the heated trypsinized sample survived without apparent illness for at least 48 hr; and, 3) mice not receiving antitoxin nor heated extract all died with symptoms of botulism within 24 hr.

RESULTS AND DISCUSSION

I. Survey of Public Health-related Bacteria in Freshwater Crayfish and Water from Various Areas

The purpose of this survey was to determine the incidence of public health-related bacteria which may occur in freshwater crayfish from their natural sources. Analysis of water for these bacteria were also performed. Cleanliness of food is considered essential by the consumer. Consumers place a trust in food producers, processors, and handlers that food presented has been produced under sanitary conditions and is as free of contamination as possible. There has been considerable controversy over types of organisms that are most indicative or can best be used in describing the sanitary quality of a food product. This is also true of the significance of numbers of such organisms. Of the suggested indices of sanitary quality of foods, three have been given the most consideration: *E. coli*, coliforms, and enterococci. The presence of large numbers of these types of organisms is not necessarily indicative of an immediate health hazard, but it does indicate lack of good sanitary practices and it raises a warning flag that conditions which brought about contamination could easily give rise to spoilage, loss of quality, and create a health hazard.

Therefore, it was essential to determine to what extent these organisms were present in crayfish before they were handled. It was also of interest to determine the incidence of these organisms in waters from which crayfish were obtained.

The presence or absence of the pathogenic species of Salmonella, Shigella, coagulase-positive staphylococci, and C. botulinum, type E toxin was also investigated in freshwater crayfish and their water sources. The methods of collecting and analyzing all samples are presented in the materials and methods of this paper. The water and crayfish collected represent samples from practically all of the commercial crayfish sources.

A total of 67 duplicate crayfish samples were analyzed quantitatively for coliforms, E. coli and fecal streptococci. These samples were also analyzed qualitatively for the presence of coagulase-positive Staphylococci, Shigella species, Salmonella species and C. botulinum, type E toxin. These data are presented in Table 1.

Coliform MPN per gram ranged from 36 to 1,100,000, however, most of the counts were over 1,100 organisms per gram. E. coli MPN per gram ranged from 3 to 29,000. There is a wide range noted in numbers of E. coli per gram of crayfish. This variation may be due to the fact that crayfish live primarily in the mud and feed mostly on all organic matter.

Table 1. Incidence of public health-related bacteria in crayfish from natural sources.

Site[a]	Sample Number[b]	Coliforms (MPN/g)	E. coli (MPN/g)	Fecal Streptococci (MPN/g)	Coagulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
1	1	110,000	1	19	negative	negative	negative	negative
	2	1,000	93	3	negative	negative	negative	negative
	3	11,000	100	29	negative	negative	negative	negative
	4	11,000	2,100	95	negative	negative	negative	negative
	5	11,000	290	95	negative	negative	negative	negative
	6	110,000	6,400	120	negative	negative	negative	negative
2	1	150	43	3	negative	negative	negative	negative
	2	110,000	750	3	negative	negative	negative	negative
	3	110,000	280	9	negative	negative	negative	negative
3	1	1,100	100	3	negative	negative	negative	negative
	2	43	9	3	negative	negative	negative	negative
	3	1,100	460	9	negative	negative	negative	negative
	4	24,000	3	16	negative	negative	negative	negative
4	1	1,100	3	16	negative	negative	negative	negative
	2	43,000	91	150	negative	negative	negative	negative
5	1	1,100	4	64	negative	negative	negative	negative
	2	460	23	120	negative	negative	negative	negative
	3	11,000	4	120	negative	negative	negative	negative
6	1	1,100	93	93	negative	negative	negative	negative
	2	110,000	750	290	negative	negative	negative	negative
	3	46,000	230	240	negative	negative	negative	negative

Table 1. (continued)

Site[a]	Sample Number[b]	Coliforms (MPN/g)	E. coli (MPN/g)	Fecal Streptococci (MPN/g)	Coagulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
7	1	1,100	4	290	negative	negative	negative	negative
	2	11,000	93	240	negative	negative	negative	negative
	3	110,000	230	460	negative	negative	negative	negative
8	1	39	3	36	negative	negative	negative	negative
	2	1,100	150	16	negative	negative	negative	negative
	3	1,100	35	3	negative	negative	negative	negative
	4	1,100	1,100	75	negative	negative	negative	negative
	5	1,100	1,100	43	positive (10^{-5})	negative	negative	negative
	6	11,000	36	93	negative	negative	negative	negative
	7	11,000	430	43	negative	negative	negative	negative
	8	4,600	750	9	negative	negative	negative	negative
	9	110,000	2,300	460	negative	negative	negative	negative
9	1	36	7	3	negative	negative	negative	negative
	2	1,100	1,100	210	negative	negative	negative	negative
	3	1,100	1,100	150	negative	negative	negative	negative
	4	110,000	2,700	460	positive (10^{-3})	negative	negative	negative
10	1	1,100	3	3	negative	negative	negative	negative
	2	1,100	9	3	negative	negative	negative	negative
	3	11,000	230	3	negative	negative	negative	negative
11	1	11,000	430	36	negative	negative	negative	negative
	2	11,000	390	3	negative	negative	negative	negative
	3	1,100,000	3,900	460	negative	negative	negative	negative

Table 1. (continued)

Site[a]	Sample Number[b]	Coliforms (MPN/g)	E. coli (MPN/g)	Fecal Streptococci (MPN/g)	Coagulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
12	1	1,100	3	3	negative	negative	negative	negative
	2	11,000	1,500	36	negative	negative	negative	negative
	3	11,000	1,100	12	negative	negative	negative	negative
13	1	1,100	39	19	negative	negative	negative	negative
14	1	1,100	460	[c]	negative	negative	negative	negative
15	1	110,000	1,100	290	negative	negative	negative	negative
	2	1,100	1,100	210	negative	negative	negative	negative
	3	110,000	390	460	negative	negative	negative	negative
16	1	11,000	460	3	negative	negative	negative	negative
	2	1,100	240	3	negative	negative	negative	negative
	3	11,000	230	460	negative	negative	negative	negative
	4	46,000	7,500	36	negative	negative	negative	negative
	5	11,000	230	240	negative	negative	negative	negative
	6	110,000	29,000	210	negative	negative	negative	negative
17	1	1,100	1.100	[c]	negative	negative	positive	negative
	2	110,000	36	[c]	negative	negative	positive	negative
18	1	1,100	240	3	negative	negative	negative	negative
	2	110,000	280	3	negative	negative	negative	negative
19	1	430	230	[c]	negative	negative	negative	negative

Table 1. (continued)

Site[a]	Sample Number[b]	Coliforms (MPN/g)	E. coli (MPN/g)	Fecal Streptococci (MPN/g)	Coaglulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
20	1	11,000	1,100	23	negative	negative	negative	negative
	2	1,100,000	930	39	negative	negative	negative	negative
21	1	11,000	4,600	39	negative	negative	negative	negative
22	1	110,000	1,100	93	negative	negative	negative	negative
	2	110,000	230	150	negative	negative	negative	negative

[a] Sites: 1 = Atchafalaya floodway in the Henderson area
2 = Atchafalaya floodway, Grand River flats
3 = Woodland pond, Wade Martin
4 = Ricefield, L. Miller
5 = Swamps near Lake Veret
6 = Swamps near Pierre Part
7 = Atchafalaya floodway, Opelousas Bay
8 = Marsh near Pecan Island
9 = Woodland pond, Elmer Naquin
10 = Ricefields near Henderson
11 = Atchafalaya floodway near Pierre Part
12 = Swamp, Grand Bayou
13 = Ricefield, John Ruppert
14 = Ricefield, Roland Faulk
15 = Atchafalaya floodway near Rosedale
16 = Atchafalaya floodway from Henderson
17 = Marsh near Forked Island
18 = Atchafalaya floodway, Bayou Pidgeon
19 = Open land (pasture) near Pecan Island
20 = Atchafalaya floodway, Belle River
21 = Atchafalaya floodway, Breaux Bridge
22 = Natural lake (Lake Pearl)

[b] Collections began February 15, 1966 and extended through June 7, 1966.

[c] Cell counts not determined.

Fecal Streptococci MPN per gram ranged from 3 to 460. These counts are somewhat lower than was found in the case of E. coli. Four samples in the fecal Streptococci survey were not analyzed. These samples were collected early in the project before the special media, which is necessary for its determination, was obtained.

In only 2 incidents of 67 were coagulase-positive staphylococci found in live crayfish. This organism was found in 10^{-5} dilutions from one area and in 10^{-3} dilutions from a different area. Both of these samples were obtained from fishermen and it is believed that this contaminate could be from handling by fishermen.

Salmonella species were found on two different occasions in live crayfish from the same water source. On both occasions this organism was detected in the water samples (Table 2). This area is one which appears to be heavily polluted. This was the only source of crayfish showing a positive test for Salmonella species of bacteria.

Shigella species and C. botulinum were not detected in any of the 67 samples.

In the water analysis survey 50 duplicate samples were analyzed for these organisms (Table 2). Coliform MPN per ml ranged from 23 to 46,000. This range was much higher in the live crayfish survey (Table 1). E. coli MPN per ml ranged from 3 to 2,300 and fecal streptococci MPN per ml ranged from 3 to 460 which was similar to ranges found in live crayfish.

Table 2. Analysis of water from various crayfish sources for public health-related bacteria.

Site[a]	Number[b]	Coliforms (MPN/ml)	E. coli (MPN/ml)	Fecal Streptococci (MPN/ml)	Coagulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
1	1	1,100	9	23	negative	negative	negative	negative
	2	460	3	39	negative	negative	negative	negative
3	1	460	3	3	negative	negative	negative	negative
	2	11,000	75	23	negative	negative	negative	negative
	3	1,100	3	3	negative	negative	negative	negative
	4	46,000	3	3	negative	negative	negative	negative
5	1	11,000	93	44	negative	negative	negative	negative
	2	1,000	93	23	negative	negative	negative	negative
	3	1,000	240	93	negative	negative	negative	negative
	4	1,000	43	9	negative	negative	negative	negative
6	1	240	9	9	negative	negative	negative	negative
	2	23,000	91	23	negative	negative	negative	negative
	3	110	36	23	negative	negative	negative	negative
7	1	1,100	460	3	negative	negative	negative	negative
8	1	23	4	3	negative	negative	negative	negative
	2	110	23	3	negative	negative	negative	negative
	3	11,000	460	23	negative	negative	negative	negative
	4	110	43	3	negative	negative	negative	negative
	5	930	15	3	negative	negative	negative	negative

Table 2. (continued)

Site[a]	Sample Number[b]	Coliforms (MPN/ml)	E. coli (MPN/ml)	Fecal Streptococci (MPN/ml)	Coagulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
9	1	230	3	3	negative	negative	negative	negative
	2	1,200	36	12	negative	negative	negative	negative
	3	4,600	460	93	negative	negative	negative	negative
	4	46,000	2,300	110	negative	negative	negative	negative
11	1	93	4	3	negative	negative	negative	negative
	2	1,100	3	3	negative	negative	negative	negative
	3	4,600	930	93	negative	negative	negative	negative
	4	1,500	210	150	negative	negative	negative	negative
13	1	1,100	93	3	negative	negative	negative	negative
	2	7,500	120	12	negative	negative	negative	negative
15	1	1,500	93	75	negative	negative	negative	negative
	2	4,600	290	460	negative	negative	negative	negative
	3	2,400	210	36	negative	negative	negative	negative
16	1	11,000	36	3	negative	negative	negative	negative
	2	1,100	43	23	negative	negative	negative	negative
	3	4,600	210	120	negative	negative	negative	negative
	4	3,900	230	93	negative	negative	negative	negative
17	1	240	4	3	negative	negative	positive	negative
	2	11,000	36	3	negative	negative	positive	negative

Table 2. (continued)

Site[a]	Sample Number[b]	Coliforms (MPN/ml)	E. coli (MPN/ml)	Fecal Streptococci (MPN/ml)	Coagulase-positive Staphylococci (present)	Shigella species (present)	Salmonella species (present)	C. botulinum (present)
18	1	1,100	460	36	negative	negative	negative	negative
	2	4,300	91	12	negative	negative	negative	negative
20	1	2,900	1,100	93	negative	negative	negative	negative
	2	4,600	93	19	negative	negative	negative	negative
21	1	930	430	110	negative	negative	negative	negative
	2	1,100	93	3	negative	negative	negative	negative
	3	4,600	1,500	93	negative	negative	negative	negative
	4	4,600	930	150	negative	negative	negative	negative
22	1	29,000	390	93	negative	negative	negative	negative
	2	1,100	9	3	negative	negative	negative	negative
	3	4,600	1,500	75	negative	negative	negative	negative
	4	21,000	460	93	negative	negative	negative	negative

a/Same as a/ in Table 1.

b/Collections began March 1, 1966 and extended through July 7, 1966.

Salmonella species were found in two water samples from the same area. All other areas were negative. Coagulase-positive Staphylococci, Shigella species, and C. botulinum, type E toxin, were not detected in any of the water sources.

Table 3 summarizes results of the incidence of coliforms, E. coli and fecal Streptococci in freshwater crayfish from natural sources. Means and 95% Confidence Limits of cell counts per gram were determined according to statistical methods of Snedecor (1962). This "95% Confidence Limit" indicates that it can be assumed that in 95% of the samples this interval will cover the true population percentage.

The average cell count for coliforms of the 67 samplings was 60,023 per gram. The 95% confidence limit is 13,931 to 106,115 coliforms per gram. These figures indicate a high coliform level in whole, live freshwater crayfish when taken from their natural sources. All samples tested contained coliforms.

The average of 67 samples examined for E. coli was 1,229.8 per gram. The 95% confidence limit is 323 to 2,137 E. coli per gram. The average fecal Streptococci per gram in 63 samples was 115.4. The 95% confidence limit is 68 to 163 fecal Streptococci per gram. In 7.4% of the samples E. coli shows less than 3 organisms per gram. In 5.9% of the samples fecal Streptococci shows less than 3 organisms per gram. According to the MPN method used less than 3 indicates that the sample contains between 0 and 3 organisms per gram.

These figures (Table 3) indicate that coliforms, E. coli and fecal Streptococci are normally found in freshwater crayfish in their natural habitat. There was a marked difference in the average number of E. coli and fecal Streptococci, the fecal Streptococci being lower.

Table 4 summarizes the results of the presence of coagulase-positive Staphylococci, Shigella species, Salmonella species and C. botulinum, type E toxin, in freshwater crayfish from natural sources. Note that coagulase-positive Staphylococci and Salmonella species were each detected twice in 67 samplings (2.99%). Shigella species and C. botulinum, type E toxin, were not detected in any of the 67 samplings. These findings strongly suggest that freshwater crayfish are not normal carriers of these pathogens.

Table 5 summarizes the number of Coliforms, E. coli and fecal Streptococci in water from various sources. Fifty samples were analyzed. The average cell count per ml for Coliforms found in commercial waters was 5,974.7. The 95% confidence limit is 3,079 to 8,871 Coliforms per ml. All samples tested contained Coliforms. The average E. coli counts were 281.4 per ml with a 95% confidence limit of 466 to 508 per ml. In 12% of the samples, fecal Streptococci were found to be less than 3 per ml. In comparing the results of Tables 3 and 5, it is found that a higher average and confidence limit for coliforms and E. coli were found in crayfish than in

Table 3. Summary of the number of coliforms, *E. coli* and fecal streptococci in freshwater crayfish from natural sources.

Criteria	Coliforms	E. coli	Fecal Streptococci
Average cell count per gram	60,023	1,229	115
Confidence limits per gram ($P < .05$)	13,931 to 106,115	323 to 2,137	68 to 163
Percent of samples showing 3 per gram	0	7.4	5.9

Table 4. Summary of the presence of Coagulase-positive Staphylococci, *Shigella* species, *Salmonella* species and *C. botulinum* in freshwater crayfish from natural sources.

Criteria	Coagulase-positive Staphylococci	Shigella species	Salmonella species	C. botulinum
No. of samples showing positive test	2 of 67	0 of 67	2 of 67	0 of 67
Percent of samples showing positive test	2.99%	0%	2.99%	0%

Table 5. Summary of the number of coliforms, *E. coli* and fecal streptococci in water from natural sources.

Criteria	Coliforms	E. coli	Fecal Streptococci
Average cell count per ml	5,975	281	487
Confidence limits per ml ($P < .05$)	3,079 to 8,871	148 to 414	466 to 508
Percent of samples showing 3 per ml	0	6.0	12.0

Table 6. Summary of the presence of coagulase-positive Staphylococci, *Shigella* species, *Salmonella* species and *C. botulinum* in water from natural sources.

Criteria	Coagulase-positive Staphylococci	Shigella species	Salmonella species	C. botulinum
No. of samples showing positive test	0 of 50	0 of 50	2 of 50	0 of 50
Percent of samples showing positive test	0%	0%	4%	0%

the water samples. However, these appeared to be a higher average and confidence limit for fecal Streptococci in the water.

Table 6 summarizes the presence of coagulase-positive Staphylococci, Shigella species, Salmonella species and C. botulinum, type E toxin, in water from various sources. Salmonella species were detected twice from the same location at different sampling times. Of the 50 samples this represents only 4% positives reported. Coagulase-positive Staphylococci, Shigella species and C. botulinum were not detected in any of the 50 samples. These results strongly indicate that these pathogens are not normally found in the waters from which most of the crayfish are obtained for commercial uses.

It is of interest that in most crayfish and water samples, Coliforms, E. coli and fecal Streptococci were found (Tables 1 and 2). These findings indicate that Coliforms, E. coli and fecal Streptococci may be expected to be found in most commercial freshwater crayfish due to presence in their water environment. Consequently, crayfish could be a source for these organisms which may be found in the processed crayfish products. Also, it may be concluded that coagulase-positive staphylococci, Shigella species, Salmonella species and C. botulinum may not be expected to be found in freshwater crayfish or in their water environment.

II. Growth Studies of Microorganisms of Public Health Significance in Crayfish Products Stored for Three Weeks at 0, 5 and 25°C

The purpose of this portion of study was to investigate the growth patterns of various public health-related microorganisms in crayfish products. This study was carried out for three weeks at storage temperatures of 0, 5 and 25°C. Total plate counts and/or MPN determinations were performed at weekly intervals.

Figure 1 shows growth patterns of E. coli in raw crayfish tail flesh. The inoculum was approximately 300 organisms per gram. At 25°C organisms reproduced steadily throughout the three-week period. At 5°C there was essentially no change in the MPN of organisms per gram at 7 days. At 14 days the count was reduced to almost 100 per gram and at the end of 21 days the count was reduced to 70 organisms per gram. At 0°C organisms decreased steadily from 300 organisms per gram at zero days to 50 organisms per gram at 21 days. These results indicated that E. coli in raw crayfish flesh stored at 0 or 5°C did not appear to increase in number but perhaps decreased slightly. Controls consisted of 20 grams of raw crayfish tailmeat which were stored with each test sample. The MPN per gram for E. coli obtained from controls were subtracted from the MPN per gram resulting from the inoculated test sample.

Figure 2 indicates similar results in sterile, cooked crayfish tailmeat. E. coli organisms were able to grow steadily at 25°C in

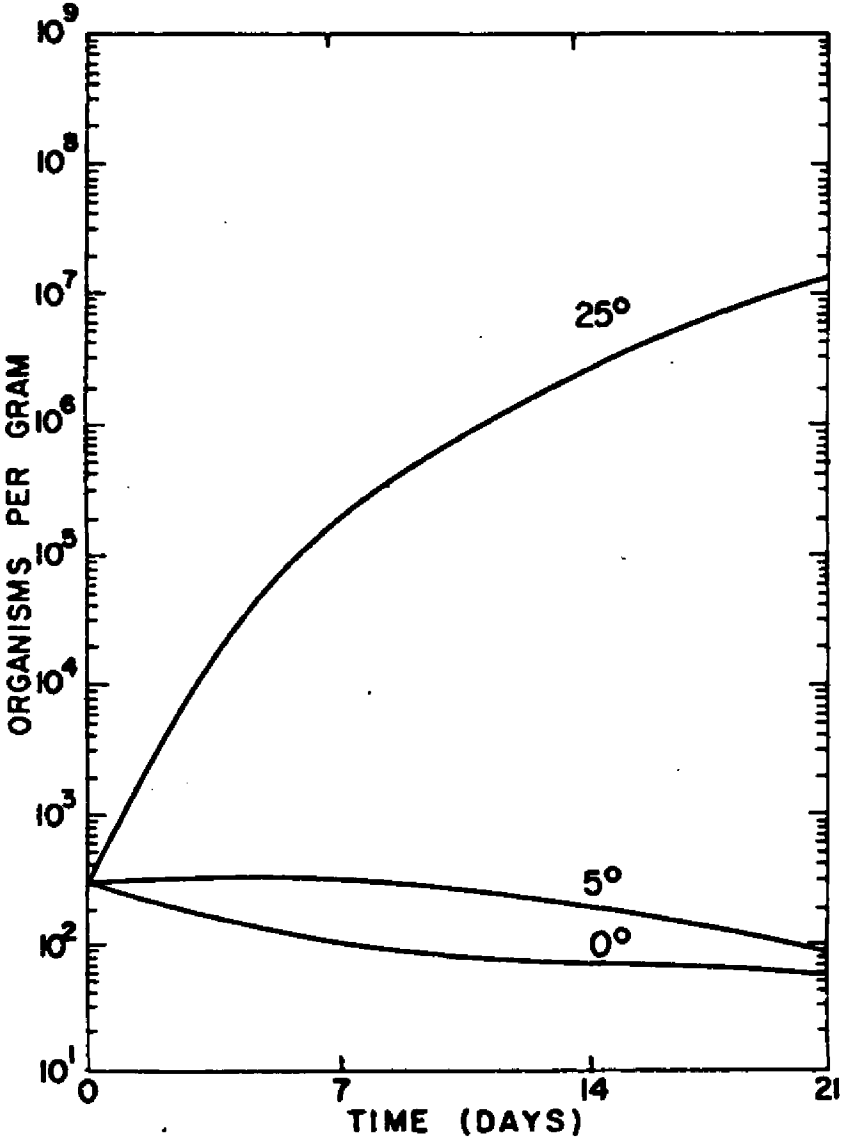

Figure 1. Three-week growth patterns of E. coli in raw tailmeat at 0, 5 and 25°C.

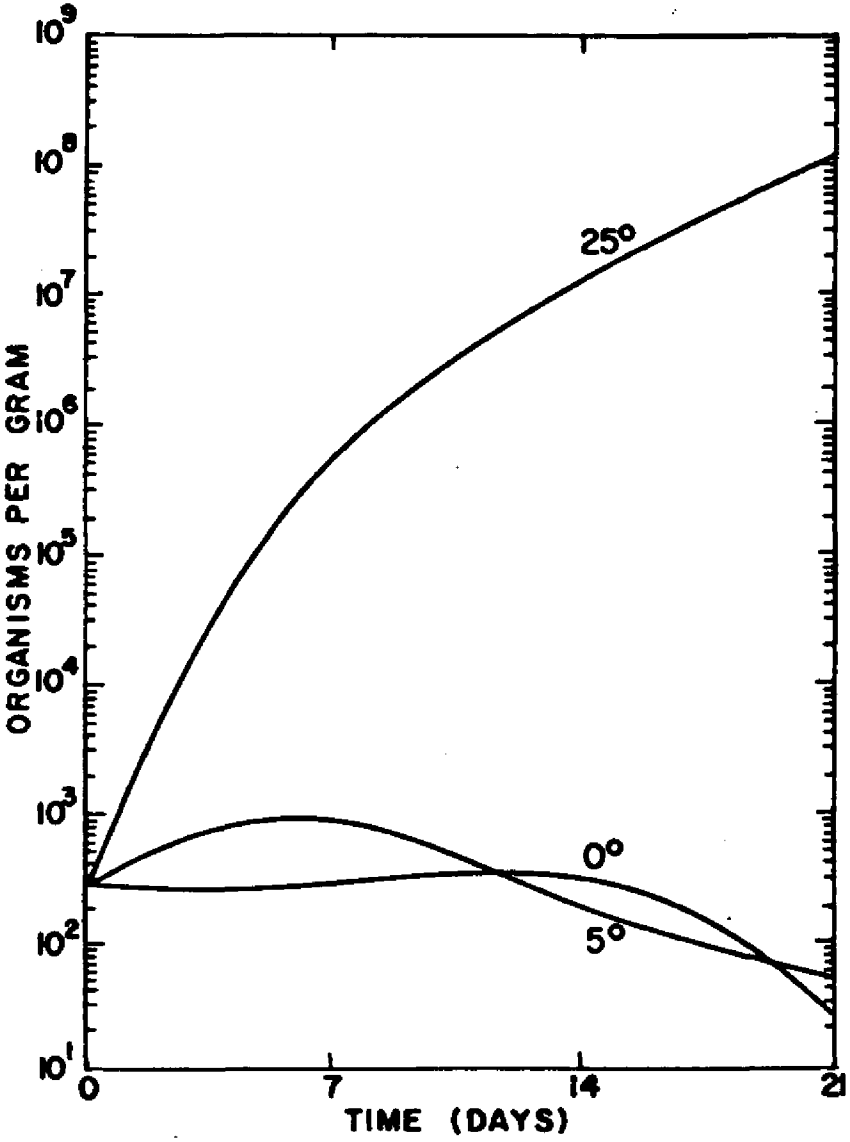

Figure 2. Three-week growth patterns of E. coli in cooked tailmeat at 0, 5 and 25°C.

a pattern similar to that in raw product, however, the count after 21 days was somewhat higher in cooked substrate. At 5°C the count increased slightly, from near 300 organisms per gram to 800 organisms per gram, in 7 days but at 14 days a decrease began to 55 organisms per gram at 21 days. At 0°C there was no apparent change in the counts through 14 days and at the end of 21 days the count dropped to 20 organisms per gram. The controls contained 20 grams of sterile, cooked crayfish tailmeat which were incubated with each test sample.

Wilson and McCleskey (1951b) found that E. coli did not multiply and usually decreased during refrigerated storage in shucked oysters. McCleskey and Boyd (1949) concluded that coliform bacteria in iced crabmeat tended to increase during the 15 day storage period. E. coli persisted throughout the sotrage period, but did not increase significantly.

As shown in Figure 3, S. typhimurium increased from 50 organisms per gram to 600 organisms per gram in raw crayfish flesh in 3 weeks at 25°C. There was a small increase shown in the count at 7 days, but thereafter the count did not increase. This response indicated that S. typhimurium grows to only a limited degree in the raw flesh at room (25°C) temperature, assuming that there was no growth inhibitory effect produced by residual microflora in the non-sterile substrate.

At 5°C there was a very slight increase at 7 days whereas in 0°C samples there was no change at 7 days. Both samples had decreased

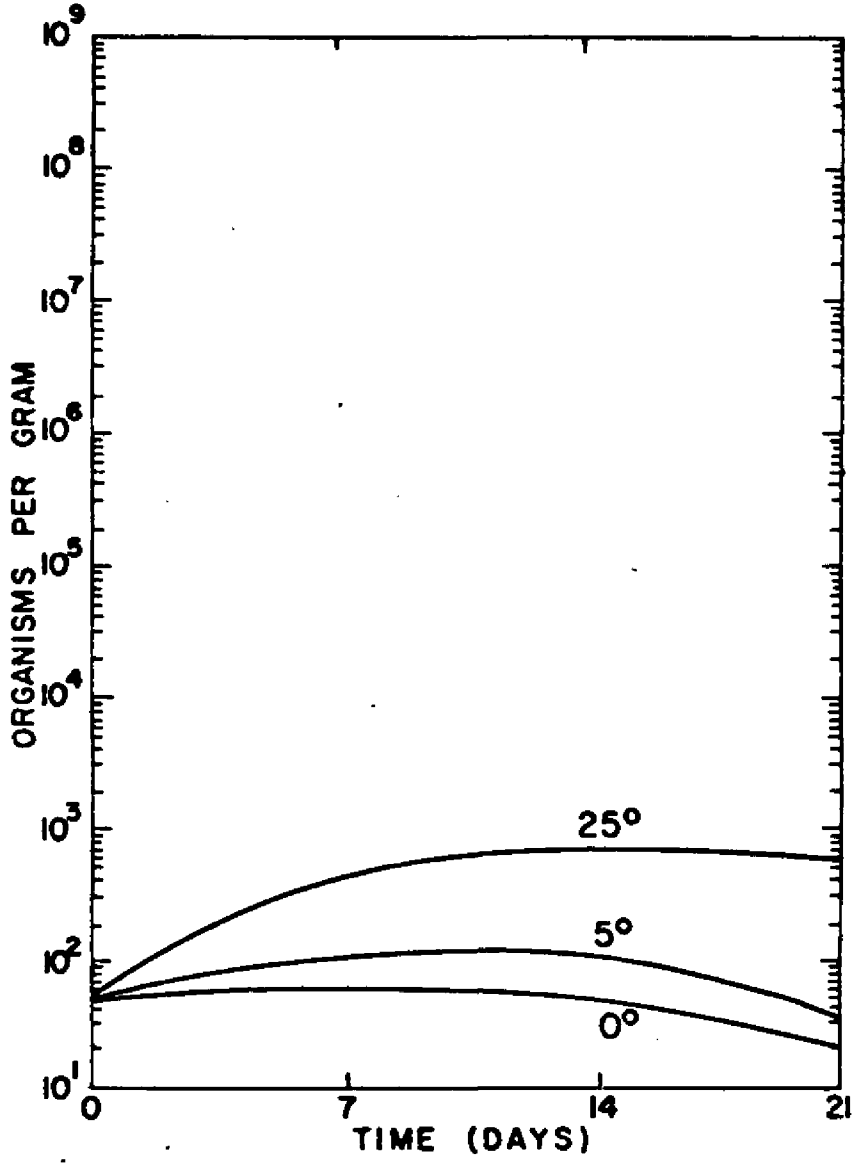

Figure 3. Three-week growth patterns of S. typhimurium in raw tailmeat at 0, 5 and 25°C.

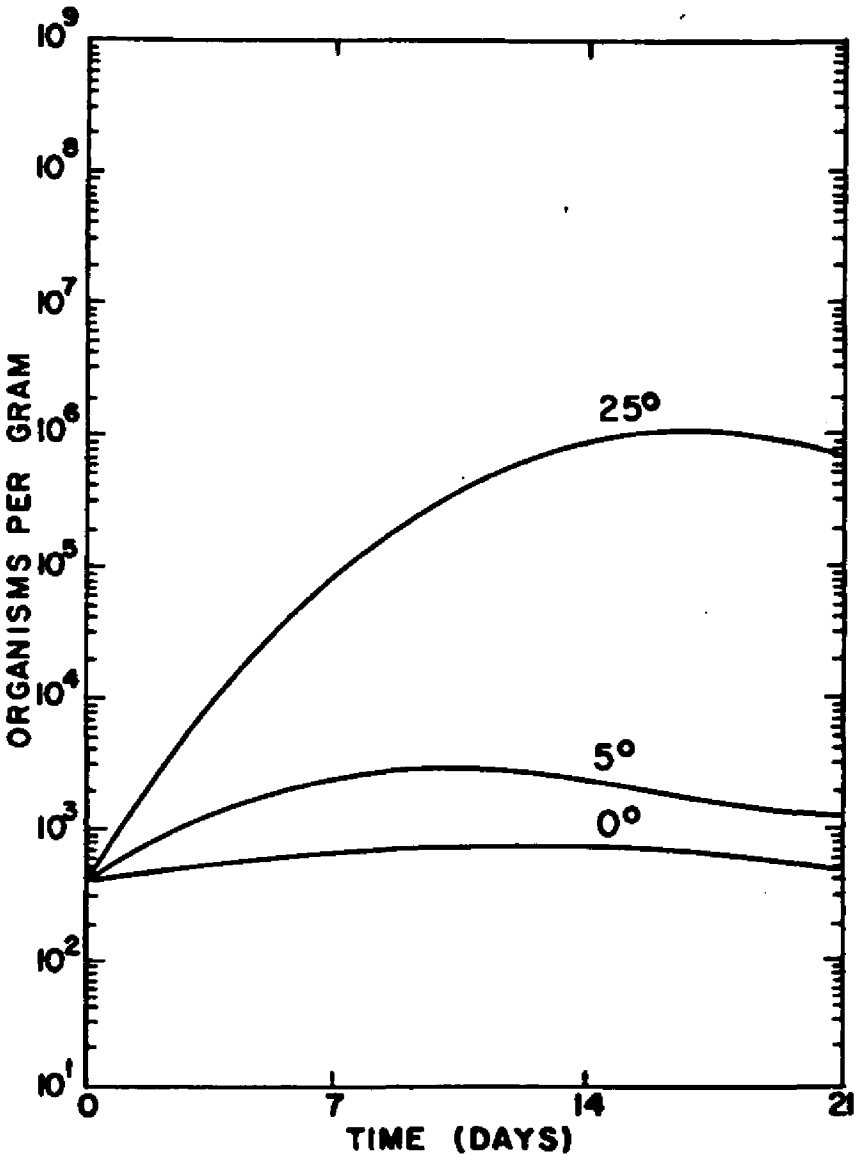

Figure 4. Three-Week growth pattern for S. typhimurium of cooked tailmeat at 0, 5 and 25°C.

slightly by 21 days. Controls consisted of 20 grams of raw crayfish flesh which were stored with each test sample.

As shown in Figure 4, S. typhimurium was able to grow considerably better at 25°C in cooked crayfish tailmeat than it did in the raw crayfish tailmeat (Figure 3). Competitive inhibition in raw tailmeat possibly explained low growth of this organism. An increase from 400 organisms to 2,000 organisms per gram was shown in 7 days at 5°C but counts then decreased to near 1,000 organisms per gram at 21 days. Counts at 0°C were essentially unchanged over a three-week period. These results are in agreement with those obtained by Kelly and Arcisz (1954b) relative to survival of S. schottmulleri in shell oysters and soft clams, stored under conditions simulating commercial practices. Organisms in both products were reduced but persisted in products for weeks at refrigeration temperatures. Controls for this study consisted of 20 grams of sterile, cooked crayfish tailmeat which were stored with each test sample.

S. dysenteriae, as shown in Figure 5, grew relatively well at 25°C in raw crayfish tailmeat. The substrate was inoculated with about 300 organisms per gram. At 14 days the count had increased to over 100,000 organisms per gram, but then decreased to 70,000 organisms per gram at 21 days. There was a slight increase in cell count at 5°C at 7 days, but at 14 days the count was reduced back to near the initial count, 300 per gram, and a further decline to 70

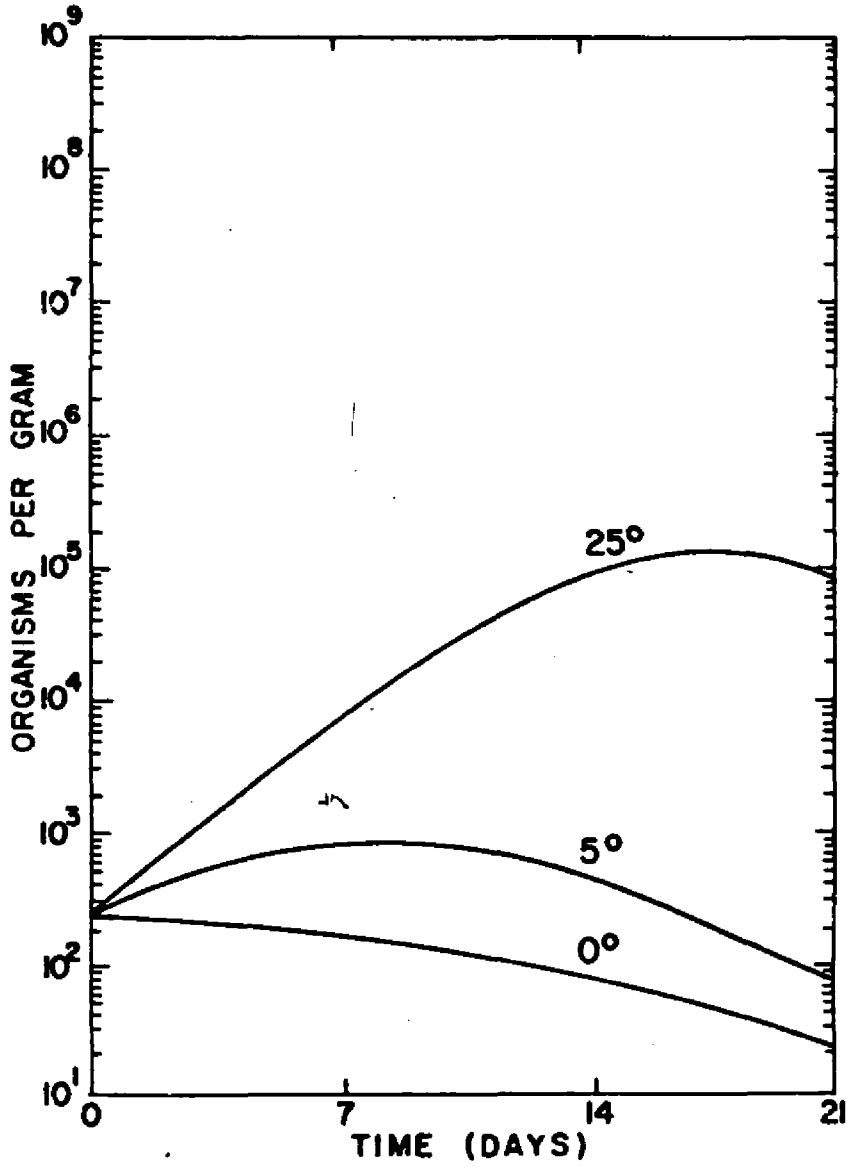

Figure 5. Three-week growth patterns of S. dysenteriae in raw tailmeat at 0, 5 and 25°C.

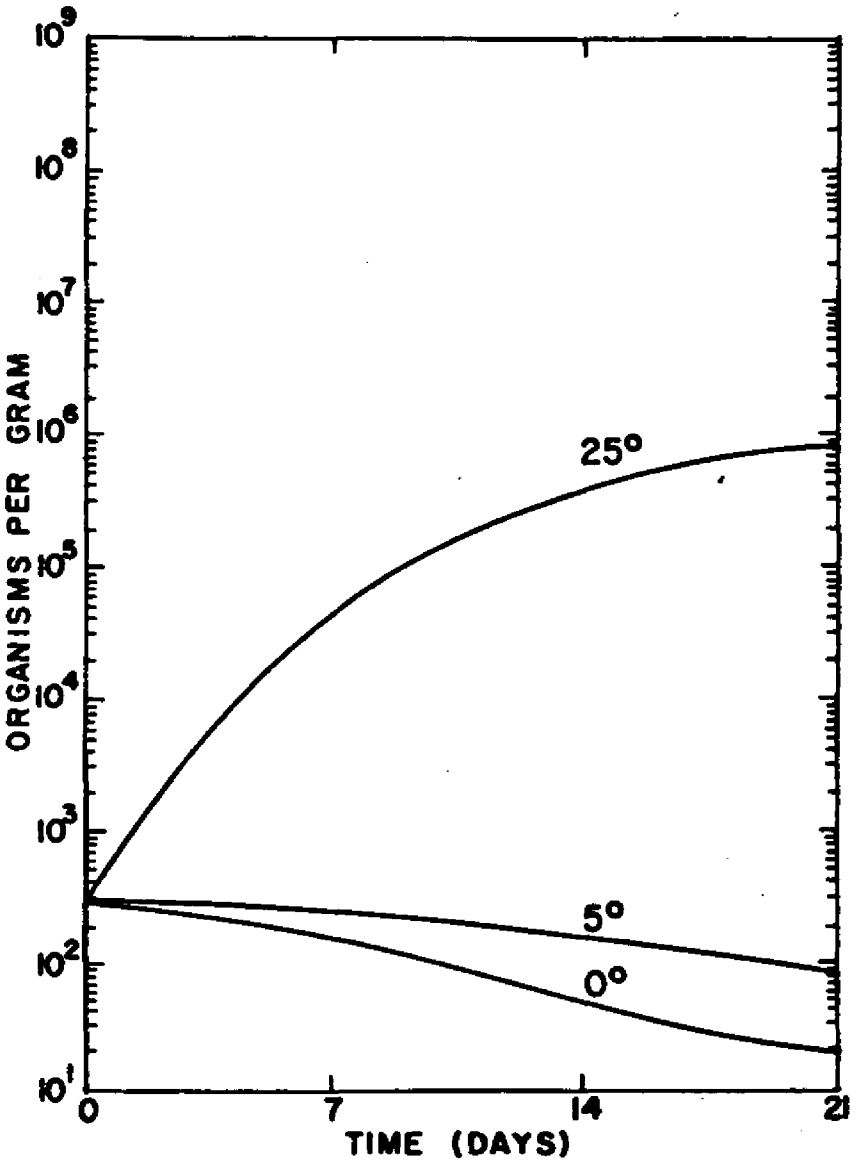

Figure 6. Three-week growth patterns of S. dysenteriae in cooked tailmeat at 0, 5 and 25°C.

organisms per gram at 21 days. There was a continuous decline in cell counts at 0°C for the 21 day period from 300 organisms per gram to 20 organisms per gram.

Similar results are shown (Figure 6) for S. dysenteriae inoculated into cooked crayfish flesh as were found in raw substrate (Figure 5) except that organisms increased to higher concentrations in the sterile, cooked flesh. At 25°C counts did not increase as was shown in the raw product. The reason for this was probably due to competitive inhibition from other naturally occurring organisms in raw flesh. However, at 5°C there was a small but steady reduction in total plate counts. At 0°C there was a more rapid decrease in counts seen after 7 days' storage. These responses indicated that S. dysenteriae do not grow well in raw or cooked crayfish flesh at refrigeration temperatures.

S. aureus did not grow well in raw crayfish flesh, even at 25°C, as is shown in Figure 7. Cell counts increased slightly, from 2,000 organisms per gram to 5,000 organisms per gram, in 7 days, then decreased to near 1,000 organisms per gram at 21 days. Apparently there was some inhibitor or toxic material present or produced by naturally occurring organisms which would cause such a low growth at 25°C. There was a very slight increase at 5°C for 7 days but counts decreased thereafter. At 0°C there was a steady decline in cell counts over the 3-week period from about 2,000 organisms per gram to about 30 organisms per gram.

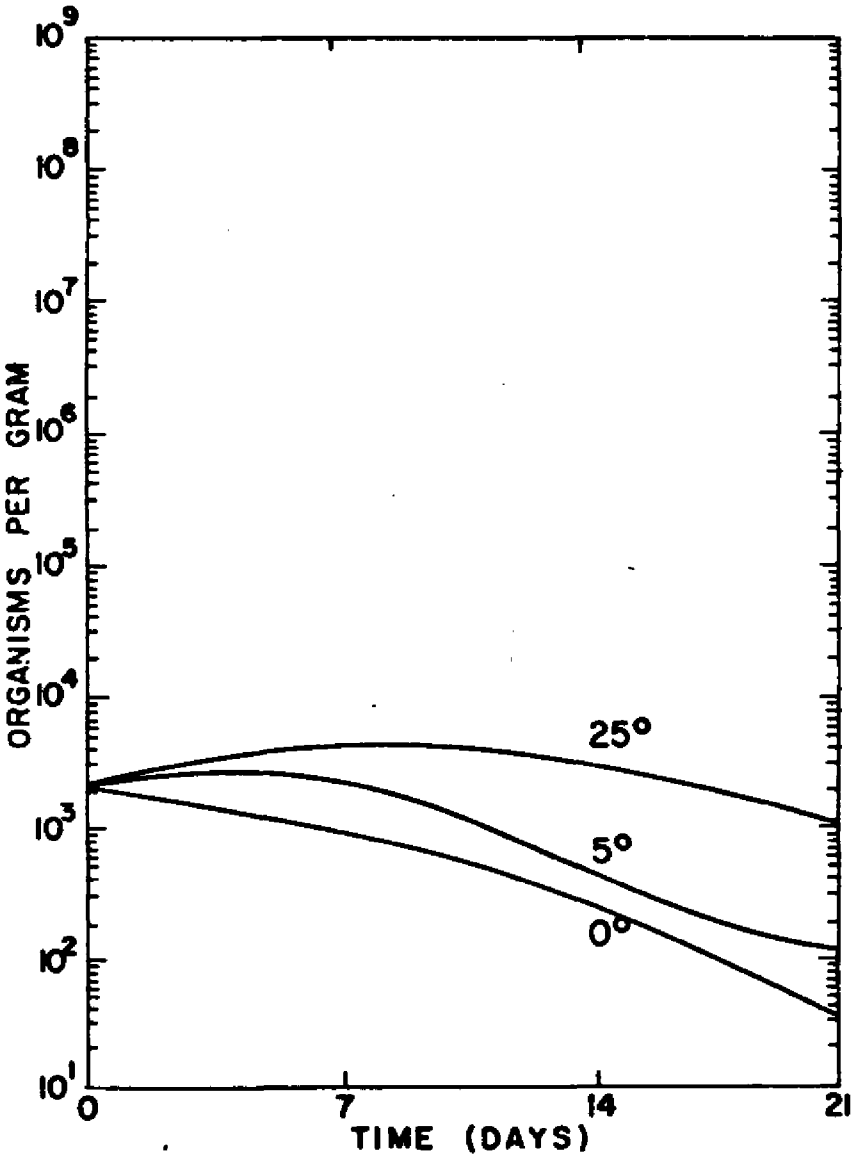

Figure 7. Three-week growth patterns of S. aureus in raw tailmeat at 0, 5 and 25°C.

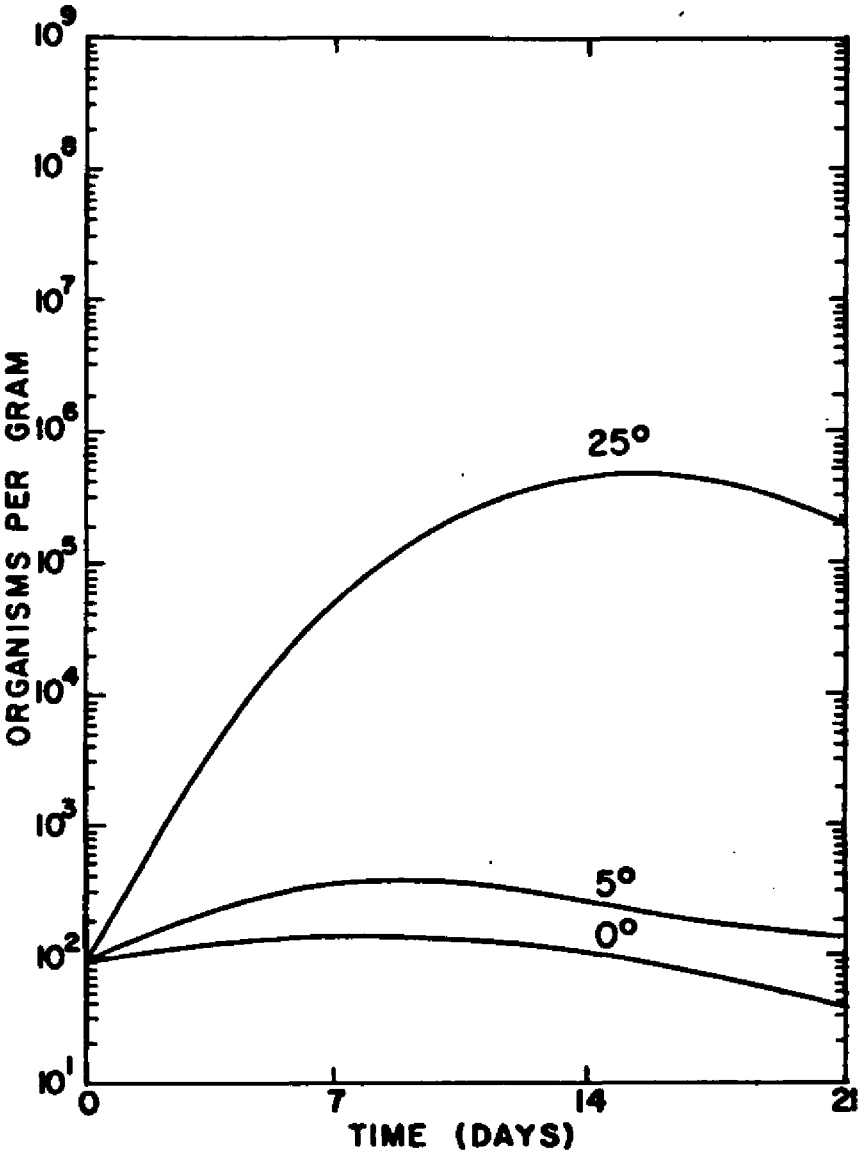

Figure 8. Three-week growth patterns of S. aureus in cooked tailmeat at 0, 5 and 25°C.

As shown in Figure 8, S. aureaus grew well at 25°C in sterile, cooked crayfish tailmeat. At 0 and 5°C there were only minor changes at 7 days, and declines in cell counts thereafter. Staphylococcus apparently does not grow significantly in raw or cooked crayfish flesh at refrigeration temperatures. Controls for this study consisted of 20 grams of sterile, cooked crayfish tailmeat stored with each of the test samples.

Figure 9 shows the growth patterns for S. fecalis in raw crayfish tailmeat. At 25°C this organism seemed to grow relatively slow at first but continued to increase throughout the 3-week period. At 5°C there was no significant change in cells per gram during the 3-week period. No change was observed at 0°C at 7 days, then a steady decline occurred for the remainder of the period. Controls contained 20 grams of raw crayfish tailmeat which were stored with each test sample.

Figure 10 shows that S. fecalis grew considerably faster in cooked crayfish tailmeat than in raw crayfish (Figure 9) at 25°C. The number of cells per gram at 7 days was 10,000 organisms per gram in raw and 100,000 organisms per gram in cooked, at 14 days they were 100,000 and 1,000,000, and at 21 days they were 600,000 and 4,000,000 for the raw and cooked, respectively. At 5°C there was an increase from 400 to 2,000 organisms per gram at 7 days and an overall increase of only 500 organisms per gram at 21 days in the cooked

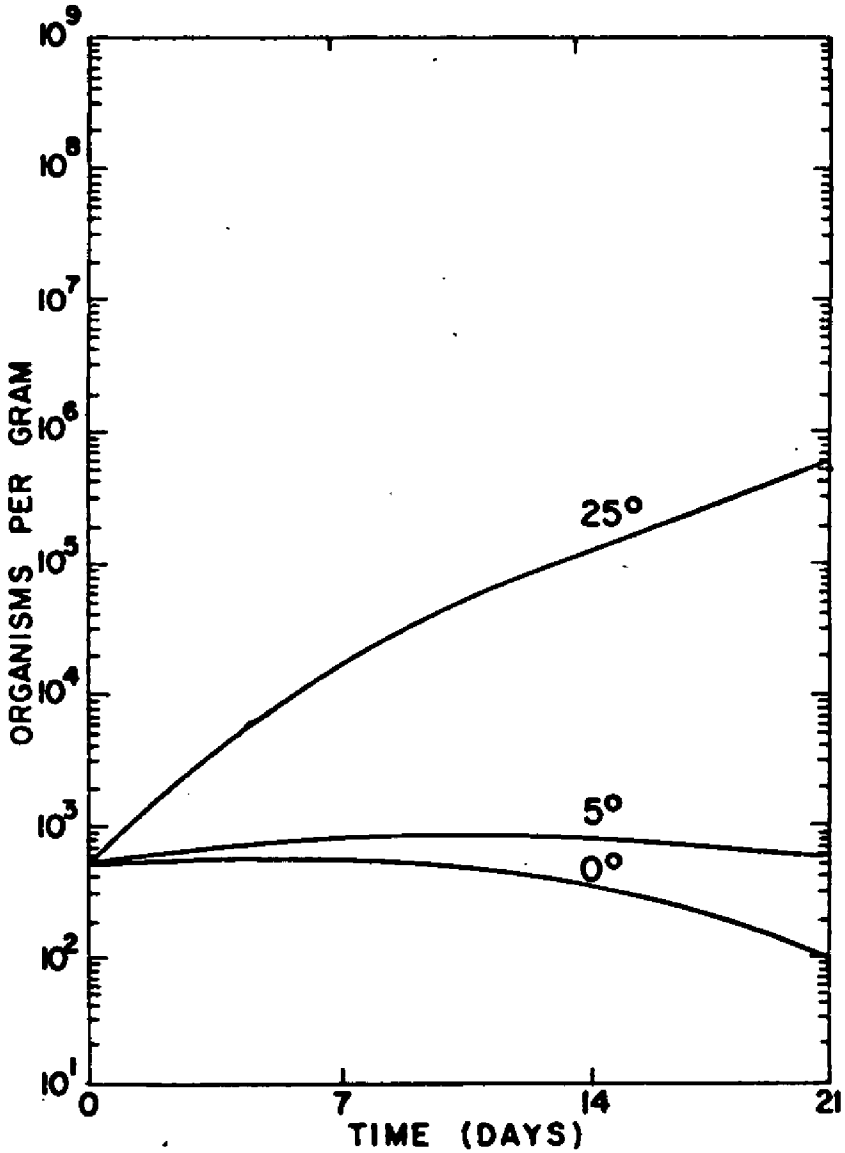

Figure 9. Three-week growth patterns of S. fecalis in raw tailmeat at 0, 5 and 25°C.

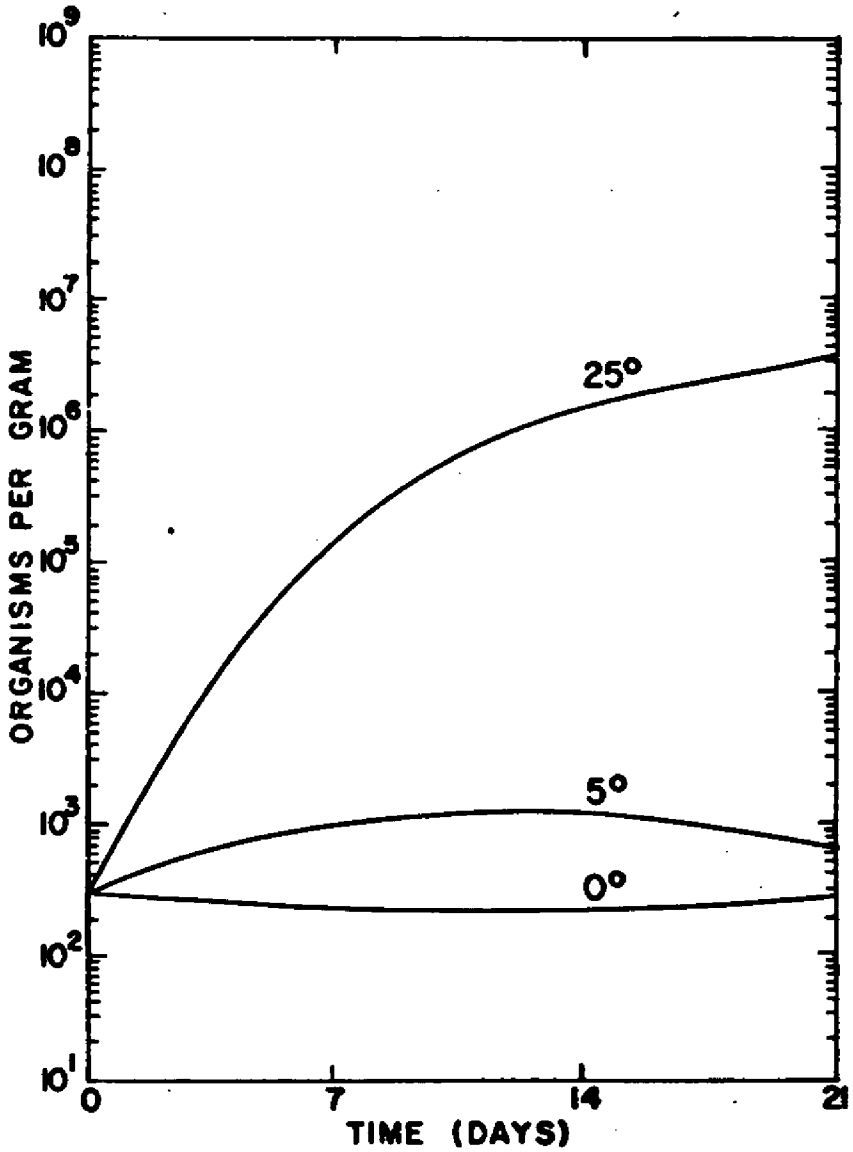

Figure 10. Three-week growth patterns of S. fecalis in cooled tailmeat at 0, 5 and 25°C.

tailmeat. There was essentially no change in cell counts at 0°C over a 3-week period.

Wilson and McCleskey (1951b) found that entercococci remained practically changed for about 3 weeks of storage at 4 to 6°C in shucked oysters. McCleskey and Boyd (1949) found no significant changes in growth of enterococci in iced crabmeat over a period of 15 days.

Results obtained in Figures 1-10 on raw and sterile, cooked crayfish tailmeat were in agreement with results obtained by Berry (1942) using sterile, cooked crabmeat. Berry found that significant increases in bacterial plate counts were observed with each culture employed when incubation temperature was 25 or 37°C. This worker also observed a decrease in cell counts at 5°C although viable organisms were still present in crabmeat after 15 days' storage. Test organisms were E. coli, Proteus sp., S. aertryske, S. dysenteriae (Flexner) and S. aureus.

Data presented indicate that the five public health-related organisms, S. typhimurium, S. dysenteriae, S. aureus, S. fecalis, and E. coli, do not grow well, at refrigerator temperatures, in raw or cooked crayfish tailmeat. At 25°C in every case the organism grew better in cooked substrate than in raw tissue. Each organism grew in cooked flesh to such a degree that it would propose a health hazard. In the case of S. aureus there was a decline in cell number and for S. typhimurium there was only minor growth in the raw flesh. Raw

flesh seemed to be an adequate substrate for growth of S. fecalis and S. dysenteriae although not equal to cooked tissue. The reason for slower relative rates of growth in raw tailmeat was probably due primarily to competitive inhibition from other naturally occurring organisms in raw, non-sterilized flesh. Antibacterial components of raw flesh could have been a factor also.

III. Early-Phase Growth Pattern Studies of Microorganisms of Public Health Significance in Crayfish Products at 5, 25 and 37°C

The purpose of this study was to investigate early phases of growth of various organisms of public health concern in raw and sterile, cooked crayfish tailmeat and a precooked crayfish product. Cell counts in three substrates were made at 0, 3, 6, 12, 18, 24, 30, 36, 48, 72, 144, and 175 hr at 5, 25 and 37°C.

Figure 11 shows growth curves for E. coli in raw crayfish tailmeat. At 37 and 25°C organisms appeared to grow well. At 37°C organisms grew rapidly for 30 hr and then began to level off. After 100 hr there was a continuous decrease noted in bacterial counts per gram. At 25°C organisms demonstrated a slower, but continuous increase in numbers. At 5°C, organisms increased for 72 hr and then counts decreased steadily. There was very little lag phase noted in samples stored at 25 and 37°C, whereas a marked lag phase of 9 hr was evident at 0°C.

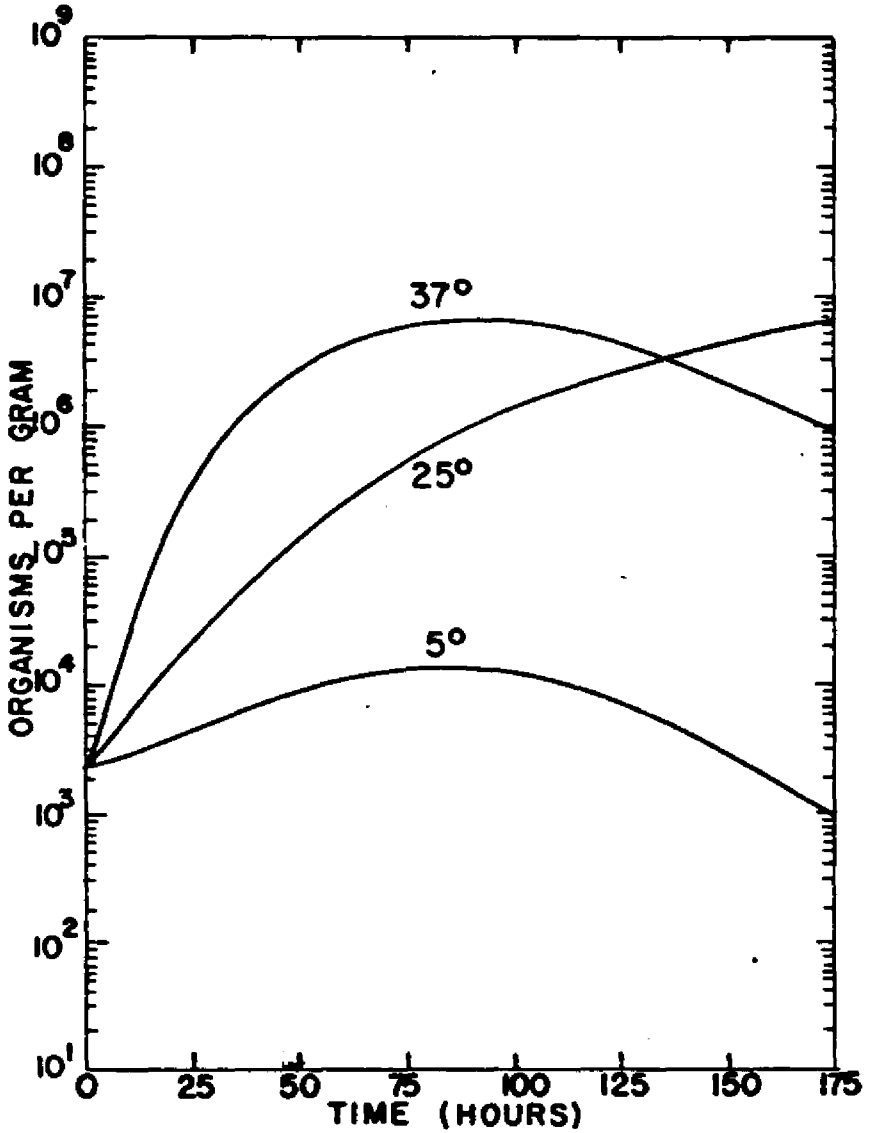

Figure 11. Growth patterns (175-hr) of E. coli in raw tailmeat at 5, 25 and 37°C.

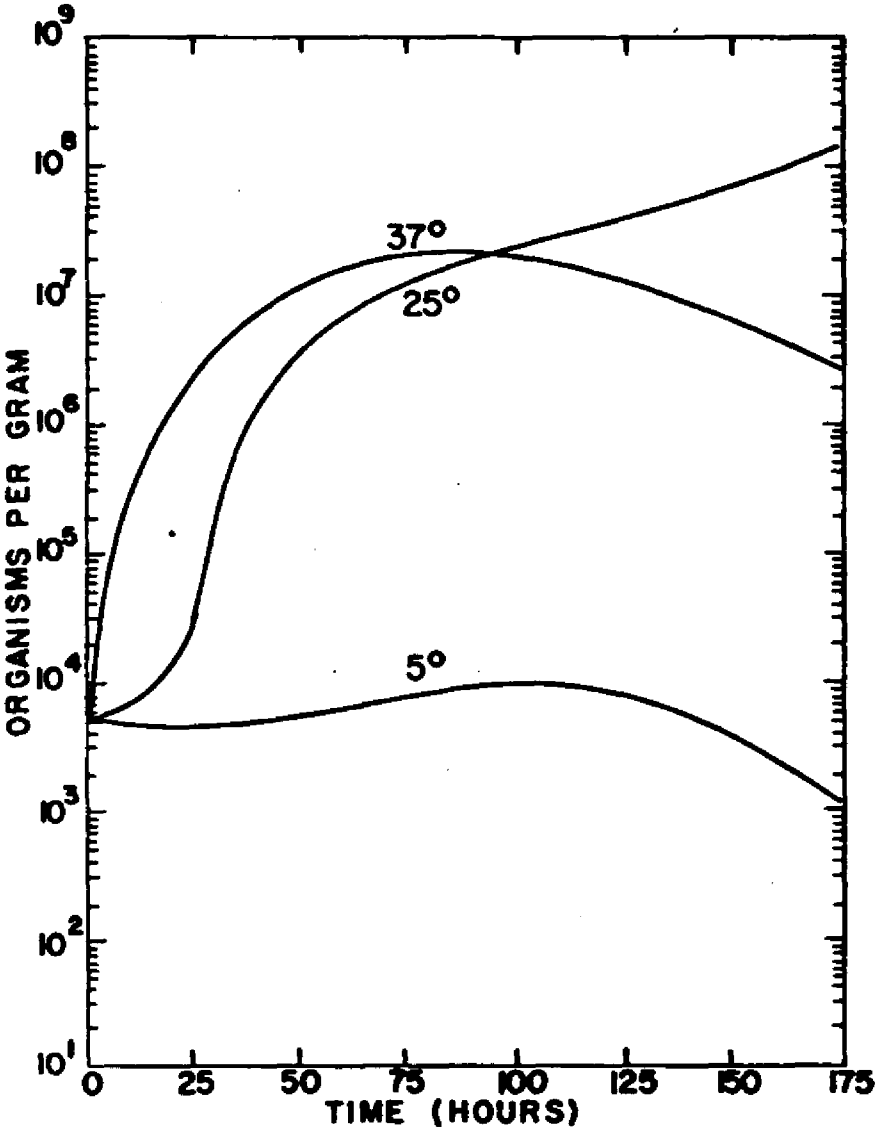

Figure 12. Growth patterns (175-hr) of E. coli in cooked tailmeat at 5, 25 and 37°C.

As shown in Figure 12, E. coli grew well in sterile, cooked crayfish tailmeat at 25 and 37°C. A longer lag phase was noted at 25°C in cooked tailmeat than in raw tailmeat as shown in Figure 11. At 5°C the total counts per gram showed a slight decrease for 36 hr, then counts increased through 100 hr and again counts began to decrease through 175 hr. The lag phase was very marked in this product as compared to raw tailmeat stored at 5°C (Figure 11).

As shown in Figure 13, E. coli grew well in crayfish etouffé at 25 and 37°C. There was an increase from 10^3 to over 10^7 organisms per gram in 36 hr. There was no apparent change in the counts after 36 hr. The lag phases at 25 and 37°C were relatively short but the lag phases were rather rapid. At 5°C the organisms increased slightly up to 36 hr and then decreased steadily through a 175-hr period. The overall reduction was from 10^3 organisms per gram at zero time to less than 2×10^2 at 175 hr. There was a marked lag phase noted with a short log phase at the end of 24 hr.

It is apparent that E. coli will grow well at high temperatures in a few hours, however, at refrigerator temperature this organism does not grow significantly for 2 or 3 days, then there is a slight increase in bacterial count. At 5°C, in all 3 products, counts began to decrease after 4 to 5 days. Apparently there was a very short lag phase in these products stored at 25 and 37°C except in the sterile, cooked tailmeat at 25°C (Figure 12).

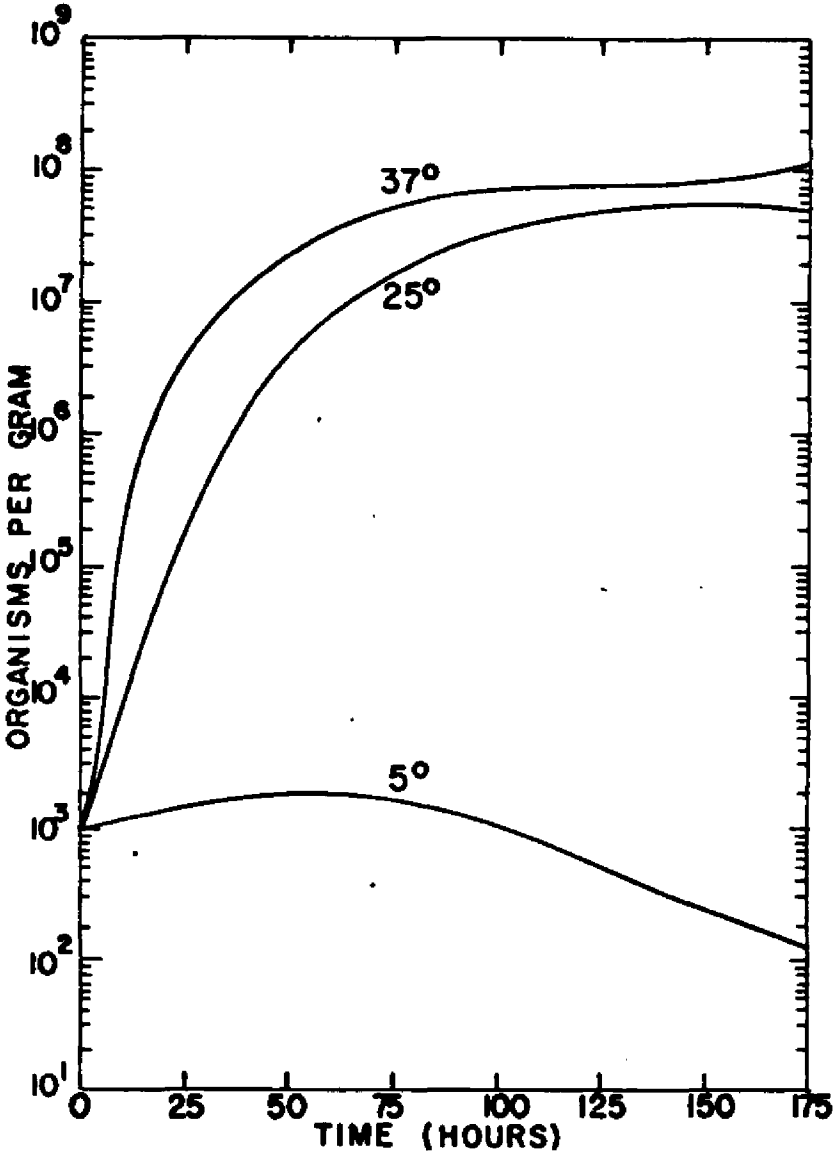

Figure 13. Growth patterns (175-hr) of E. coli in etouffé at 5, 25 and 37°C.

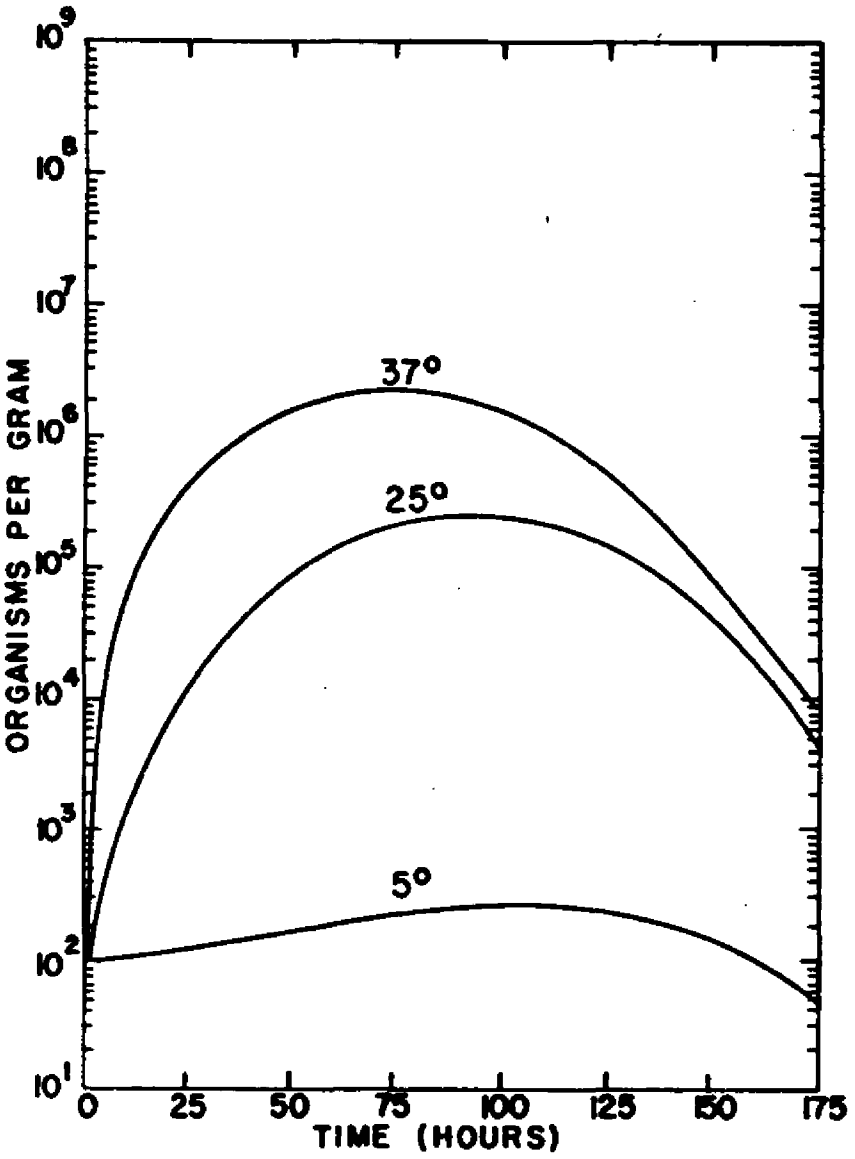

Figure 14. Growth patterns (175-hr) of S. typhimurium in raw tailmeat at 5, 25 and 37°C.

Figure 14 shows growth patterns of S. typhimurium in raw crayfish tailmeat at 5, 25 and 37°C. At 37°C organisms grew rapidly from approximately 10^2 to over 10^6 organisms per gram in 36 hr. At 72 hr counts began to decrease rapidly reaching 10^4 organisms per gram at the end of 175 hr. At 25°C growth was not as rapid, but it did show good growth. The peak growth of 2×10^5 organisms per gram was reached at 72 hr. A rapid reduction to 5×10^3 organisms per gram was demonstrated at the end of a 175-hr study. The lag periods at 25 and 37°C were very short and the log phases very rapid, especially at 37°C. At 5°C a slight increase in number of organisms was shown up to 100 hr. At this point counts decreased from 3×10^2 organisms per gram to 80 at 175 hr. A long lag phase was noted up to 24 hr followed by a slight increase in count.

Figure 15 shows growth patterns of S. typhimurium in sterile, cooked crayfish tailmeat at 5, 25 and 37°C. At 37°C counts increased from 2×10^2 organisms per gram to over 10^6 in 72 hr. At 25°C counts increased from 2×10^2 to 10^6 organisms per gram in 125 hr and finally to over 3×10^6 at 175 hr. At 5°C there was no marked change in cell count for 6 hr but at 12 hr counts increased from 2×10^2 to approximately 4×10^2 organisms per gram and decreased to about 2×10^2 per gram at 24 hr. At this point counts steadily decreased to 50 ogranisms per gram at 175 hr. The cause of this rapid increase and decrease indicates that some biochemical changes take place, up to 12 hr, which are unfavorable to this organism.

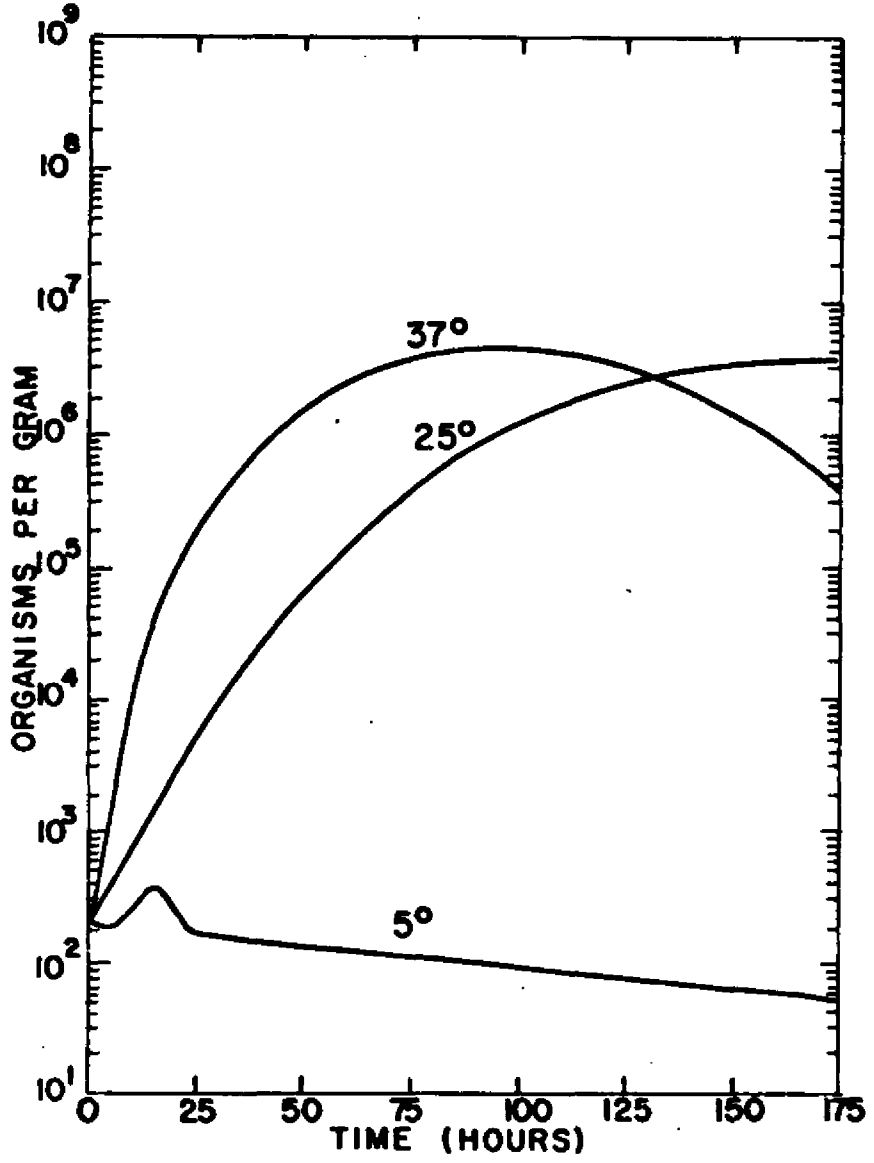

Figure 15. Growth patterns (175-hr) of S. typhimurium in cooked tailmeat at 5, 25 and 37°C.

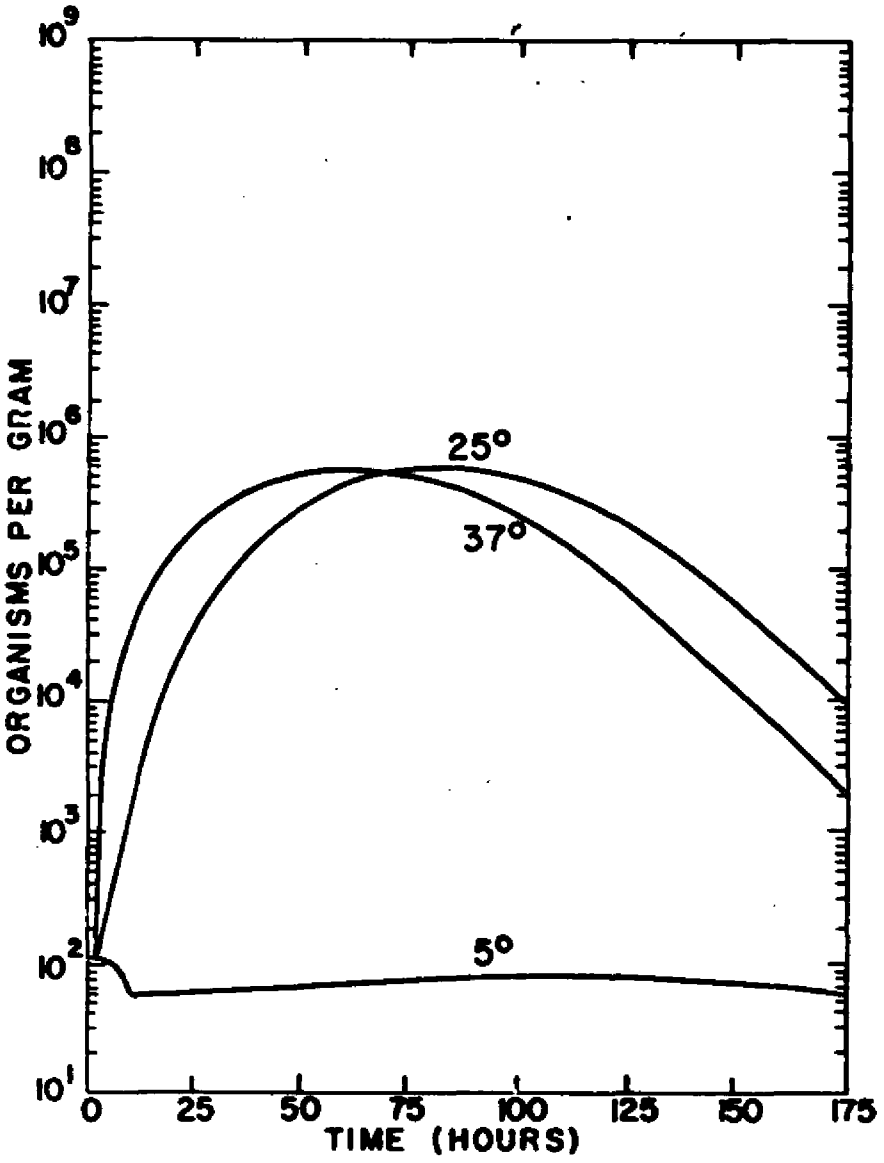

Figure 16. Growth patterns (175-hr) of S. typhimurium in etouffé at 5, 25 and 37°C.

Figure 16 shows growth patterns of S. typhimurium on crayfish etouffé at 5, 25 and 37°C. There was a much faster increase in the population at 25 and 37°C in the etouffé than was shown in raw (Figure 14) and cooked (Figure 15) crayfish tailmeat. A possible explanation for this could be the additional nutritional foods in the etouffé. In etouffé the counts increased from 10^2 to 10^6 organisms per gram in 48 hr at 37°C. There was a rapid reduction in the counts at this point which resulted in a count of 10^4 organisms per gram at 175 hr. At 5°C there was no apparent change in the population for 6 hr, then counts decreased from 10^2 to 60 organisms per gram in 9 hr. From this time to the end of the study there was no significant change in numbers of organisms per gram. This indicates that crayfish etouffé at 5°C was not a good medium for growth of this organism.

Raw crayfish tailmeat (Figure 14) showed a slight increase of organisms at 5°C over a period of 2 or 3 days whereas cooked tailmeat (Figure 15) showed a marked increase and decrease in growth in a 12 to 15 hr period and etouffé (Figure 16) showed a marked decrease in population in 6 hr.

As shown in Figure 17, S. dysenteriae grew well at 25 and 37°C in raw crayfish tailmeat. At 37°C counts increased from 10^4 to 10^6 organisms per gram in 72 hr. There was no marked change noted up to 175 hr. There was a slightly longer lag phase than was noted at 37°C. At 5°C the organism increased from 10^4 to 2×10^4 organisms per gram

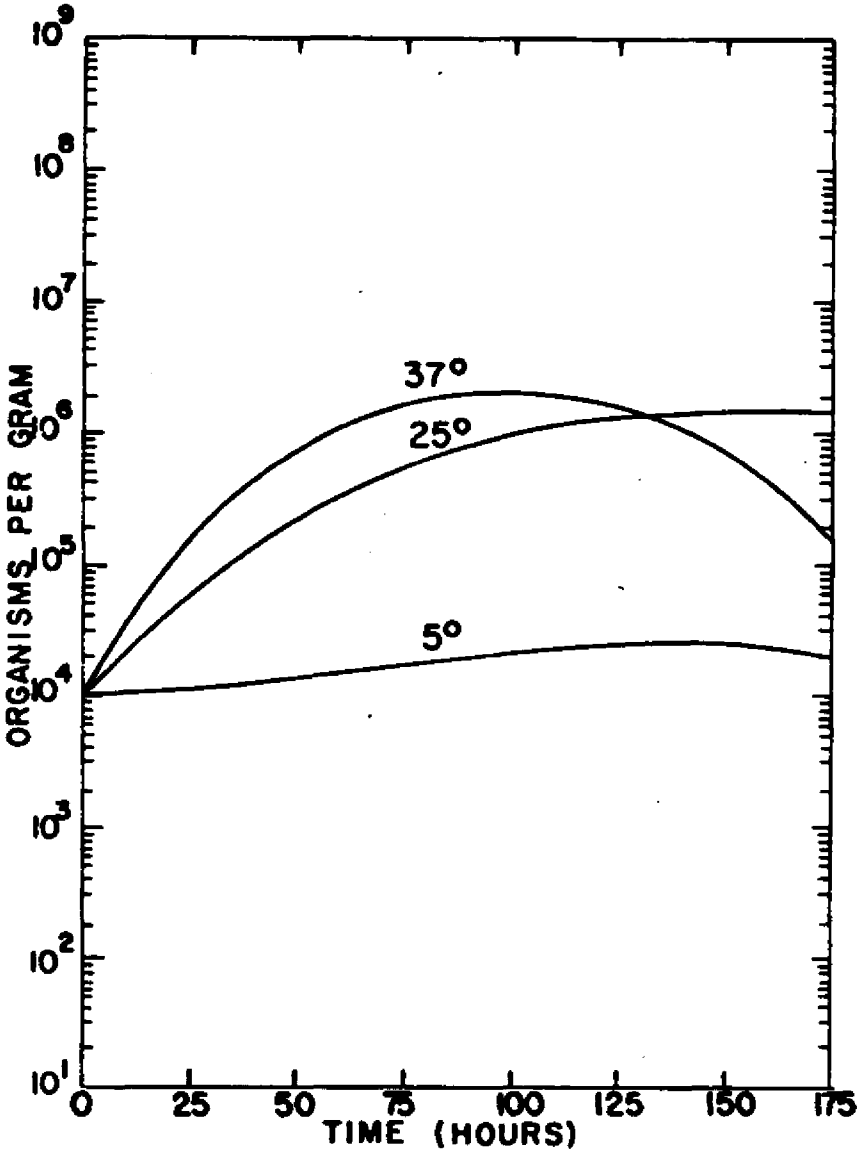

Figure 17. Growth patterns (175-hr) of S. dysenteriae in raw tailmeat at 5, 25 and 37°C.

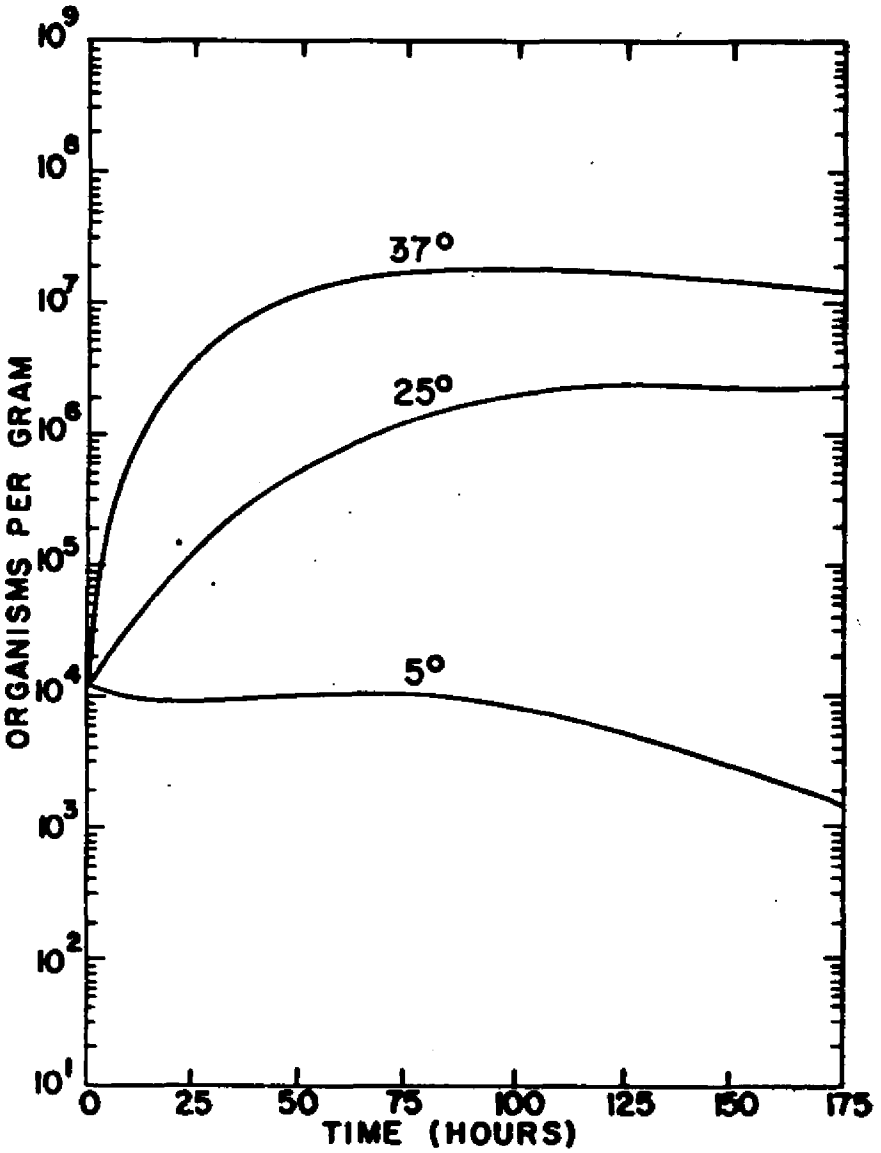

Figure 18. Growth patterns (175-hr) of S. dysenteriae in cooked tailmeat at 5, 25 and 37°C.

in 175 hr. This indicates that organisms are able to grow at 5°C in raw crayfish in the presence of other organisms. The lag phase was very long followed by a slight increase in count beginning at 36 hr.

Figure 18 shows the behavior of S. dysenteriae in sterile, cooked crayfish tailmeat at 5, 25 and 37°C. In 24 hr counts increased from approximately 10^4 to over 2×10^6 organisms per gram at 37°C. The lag phase was apparently very short with a very rapid log phase. There was no marked chage noted in counts after 48 hr. At 25°C counts increased steadily from about 10^4 to 10^6 organisms per gram in 72 hr. The lag phase was significantly longer than that noted at 37°C. At 5°C counts decreased from over 10^4 to 8×10^3 organisms per gram in 9 hr. Counts increased slightly at 36 hr and steadily decreased to 2×10^3 organisms per gram at 175 hr. This indicates that this organism is not able to grow well, if perhaps not at all, at 5°C.

Figure 19 indicates that S. dysenteriae grew quite well at 25 and 37°C in sterile crayfish etouffé. At 37°C the population increased from approximately 3×10^3 to 10^6 organisms per gram in 36 hr with a very short lag phase. There was a steady increase in the counts to 6×10^6 organisms per gram at 175 hr. There was a marked difference in the lag and log phase at 25 and 37°C. At 5°C counts did not show a marked change up to 9 hr. However, at 12 hr counts decreased from 3×10^3 to 2×10^3 organisms per gram. At 24 hr counts increased again to 3×10^3 organisms per gram and then began to decrease steadily to approximately

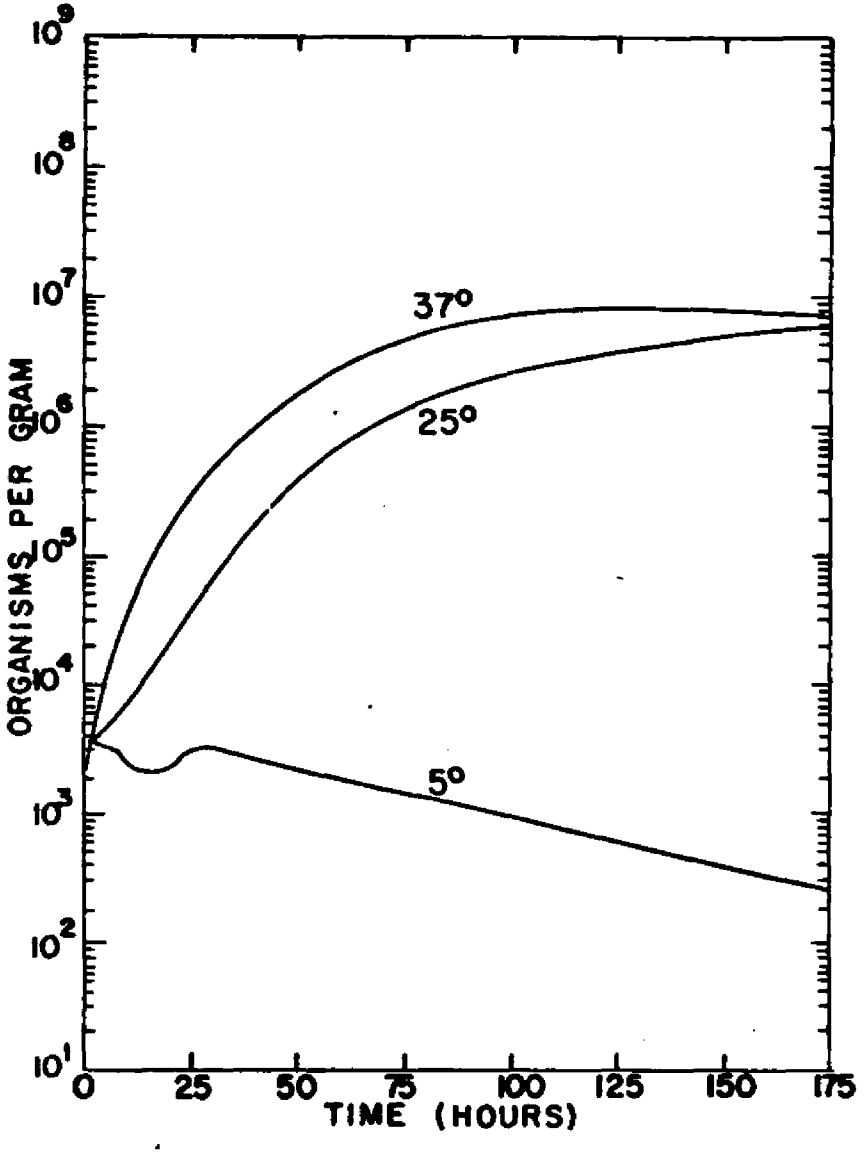

Figure 19. Growth patterns (175-hr) of S. dysenteriae in etouffe' at 5, 25 and 37°C.

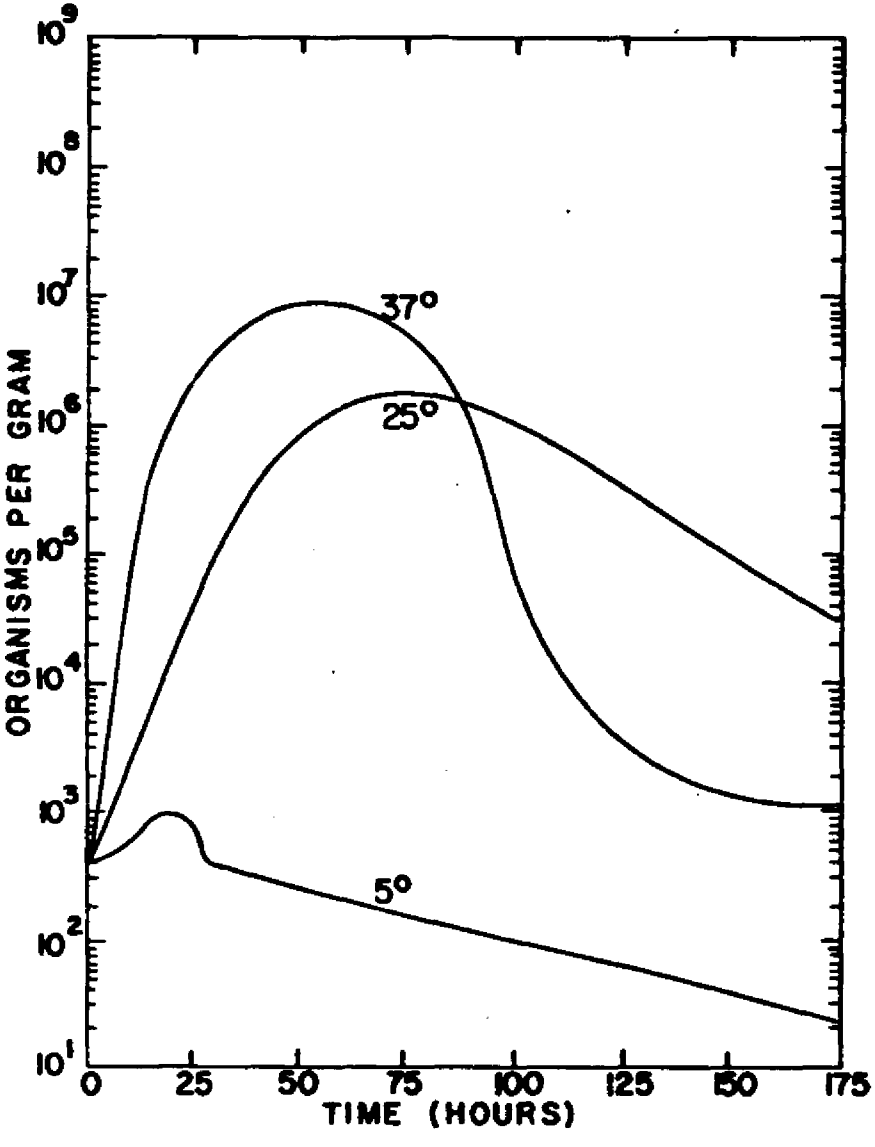

Figure 20. Growth patterns (175-hr) of S. aureus in raw tailmeat at 5, 25 and 37°C.

3×10^2 organisms per gram at 175-hr. These results strongly suggest that growth of this organism is retarded in etouffé stored at 5°C.

Figures 17, 18 and 19 indicate that S. dysenteriae grew well at 25 and 37°C in raw and cooked crayfish tailmeat and etouffé. However, at 5°C, it appeared (Figure 17) that this organism could reproduce in raw crayfish tailmeat in the presence of other microorganisms. A likely explanation for this is that other organisms present in the raw tailmeat has made available certain growth requirements for S. dysenteriae. In sterile, cooked crayfish tailmeat (Figure 18) and sterile etouffé (Figure 19) this organism did not appear to grow but decreased at 5°C.

Figure 20 illustrates that S. aureus is capable of reproducing in raw crayfish tailmeat at 25 and 37°C. At 37°C counts increased from 4×10^2 to 10^7 organisms per gram at 36 hr with a short lag phase. However, there was a rapid reduction in population, to 9×10^2 organisms per gram at 144 hr. At 25°C counts increased from 4×10^2 to 2×10^6 organisms per gram at 72 hr then counts decreased to 10^3 at 144 hr. At 5°C there was a slight decrease in population from 9 hr, then counts increased from 4×10^2 to 2×10^2 organisms per gram at 18 hr. At this point counts began to decrease steadily to 20 organisms per gram at 175-hr. This indicates that refrigeration storage temperature decreases the growth of S. aureus in raw tailmeat. Competitive growth of other organisms possibly explain this situation.

S. aureus appeared to grow well in sterile, cooked crayfish tailmeat at 25 and 37°C as shown in Figure 21. At 37°C counts increased sharply from 4×10^3 to 4×10^7 organisms per gram at 24 hr. Counts decreased to 10^6 organisms per gram at 100 hr and no marked changes were noted through the rest of the 175-hr period. At 25°C counts increased from 4×10^3 to 8×10^7 organisms per gram at 72 hr. There was a steady decrease in counts noted at 144 hr, from 8×10^7 to 3×10^6 organisms per gram at 175 hr. At 5°C there was a decrease in bacterial counts from 4×10^3 to 2×10^3 organisms per gram in 24 hr. Counts then began to increase steadily to 2×10^3 organisms per gram at 175 hr. These results strongly indicate that S. aureus is capable of growing, when not in competition with other organisms, in sterile, cooked tailmeat at refrigeration temperature.

Figure 22 indicates that S. aureus grew well in sterile crayfish etouffé at 25 and 37°C. At 37°C counts increased from 6×10^2 to 2×10^5 organisms per gram at 24 hr. After this time counts continued to increase steadily to 2×10^7 organisms per gram at 72 hr. Counts then increased slowly to 3×10^7 organisms per gram at 175 hr. At 5°C there was a decrease in counts from 6×10^2 to 3×10^2 organisms per gram at 24 hr. At this point counts began to increase steadily to 8×10^3 organisms per gram at 175 hr. This indicates that growth of this organism is enhanced in the absence of other organisms at 5°C.

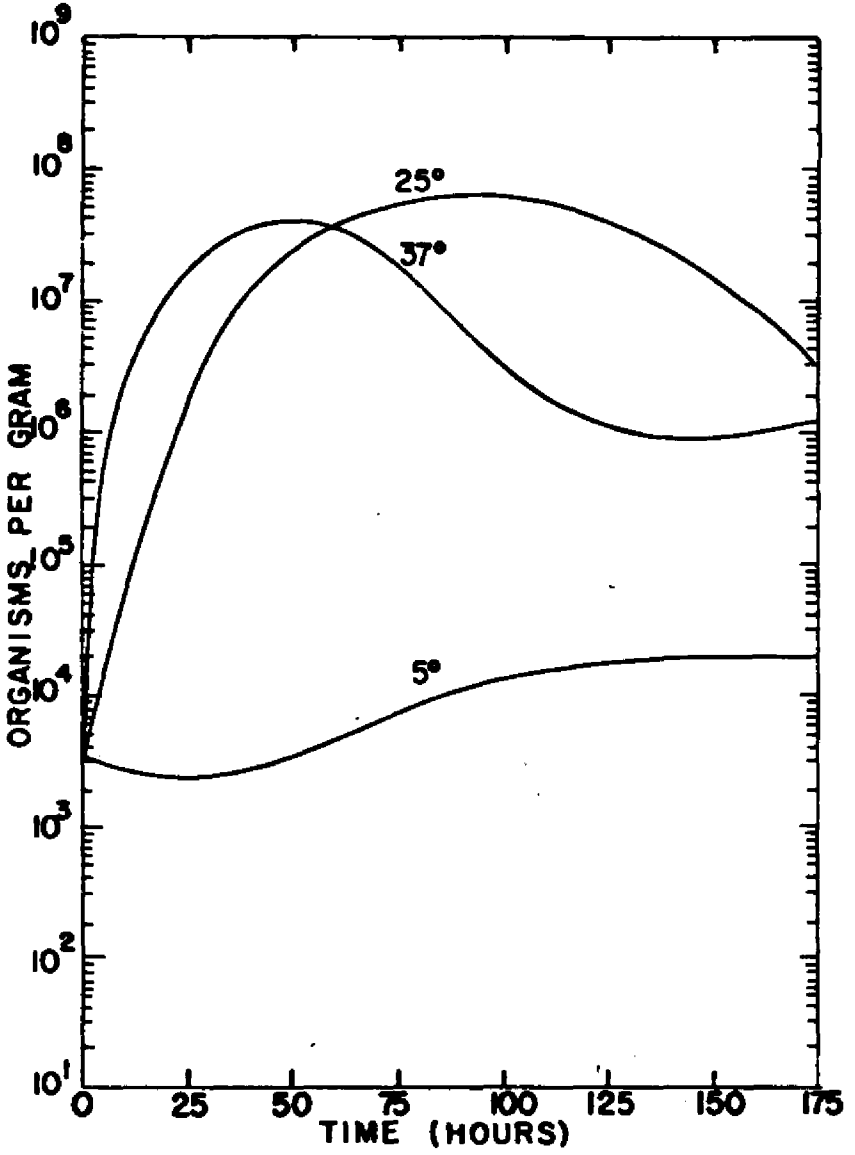

Figure 21. Growth patterns (175-hr) of S. aureus in cooked tailmeat at 5, 25 and 37°C.

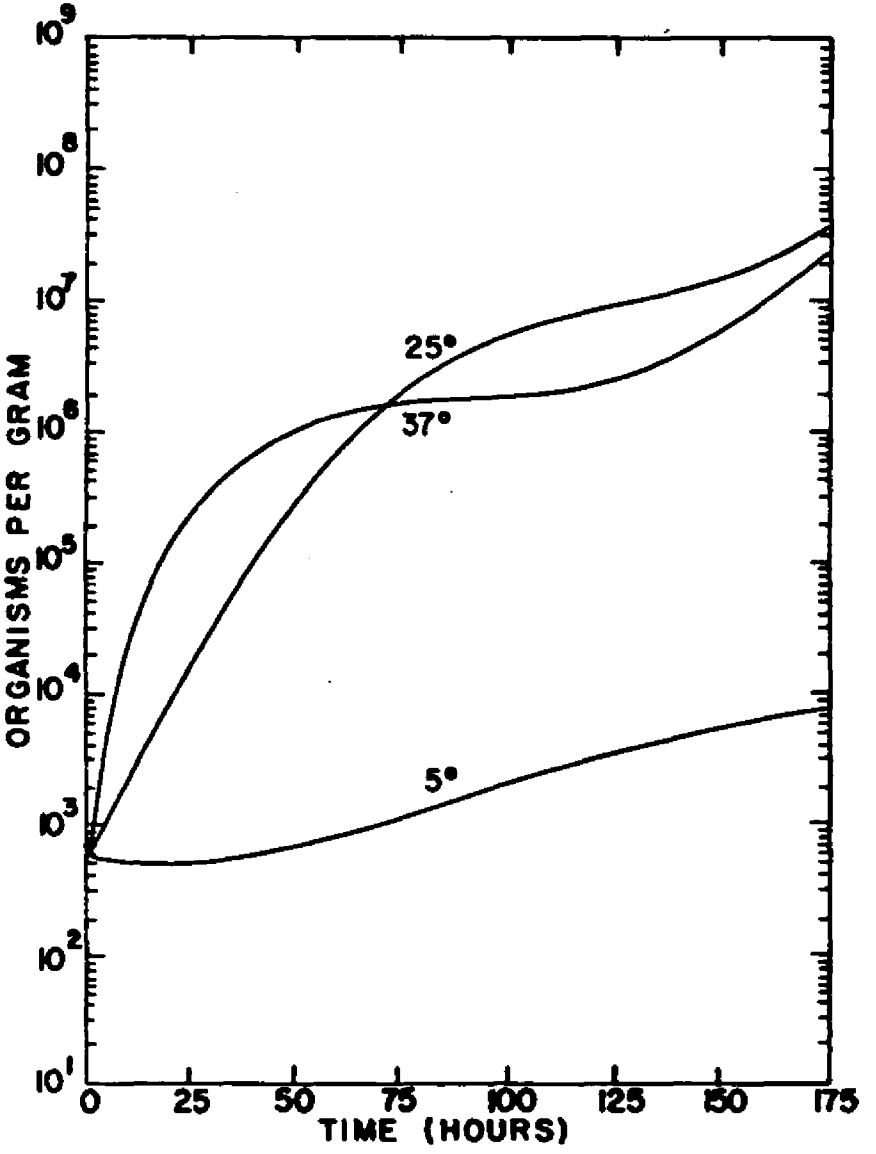

Figure 22. Growth patterns (175-hr) of S. aureus in etouffé at 5, 25 and 37°C.

It appears that S. aureus grew well for about 48 hr in raw crayfish tailmeat (Figure 20) and sterile, cooked crayfish tailmeat (Figure 21) at 25 and 37°C. The population was reduced after about 2 days probably because of depletion of food or toxic by-products released in the medium. Figure 20 indicates that S. aureus grew little in 24 hr then decreased appreciably at 5°C over a 175 hr period. Figures 21 and 22 suggest that this organism can reproduce at refrigeration temperature after 24 hr in sterile, cooked crayfish tailmeat and etouffé. Figure 22 indicates that crayfish etouffé supports growth of this organism at 25 and 37°C, longer than raw tailmeat (Figure 20) and the sterile, cooked tailmeat (Figure 21).

Figure 23 shows growth patterns of S. fecalis in raw crayfish tailmeat at 5, 25 and 37°C. It appears that this organism does not grow well when compared to the other organisms studied at 25 and 37°C. At 37°C counts increased from 3×10^2 organisms per gram to 3×10^4 in 72 hr. This count began to decrease at 144 hr to 2×10^4 organisms per gram at 175 hr. At 25°C counts increased steadily from 3×10^2 to over 10^4 organisms per gram in 144 hr. There was a short lag phase at both temperatures followed by a slow log phase. Counts showed a slight decrease at 175 hr. There was a slight increase in population noted at 72 hr.

Figure 24 indicates that S. fecalis grew well at 25 and 37°C in sterile, cooked crayfish tailmeat. At 37°C counts increased from

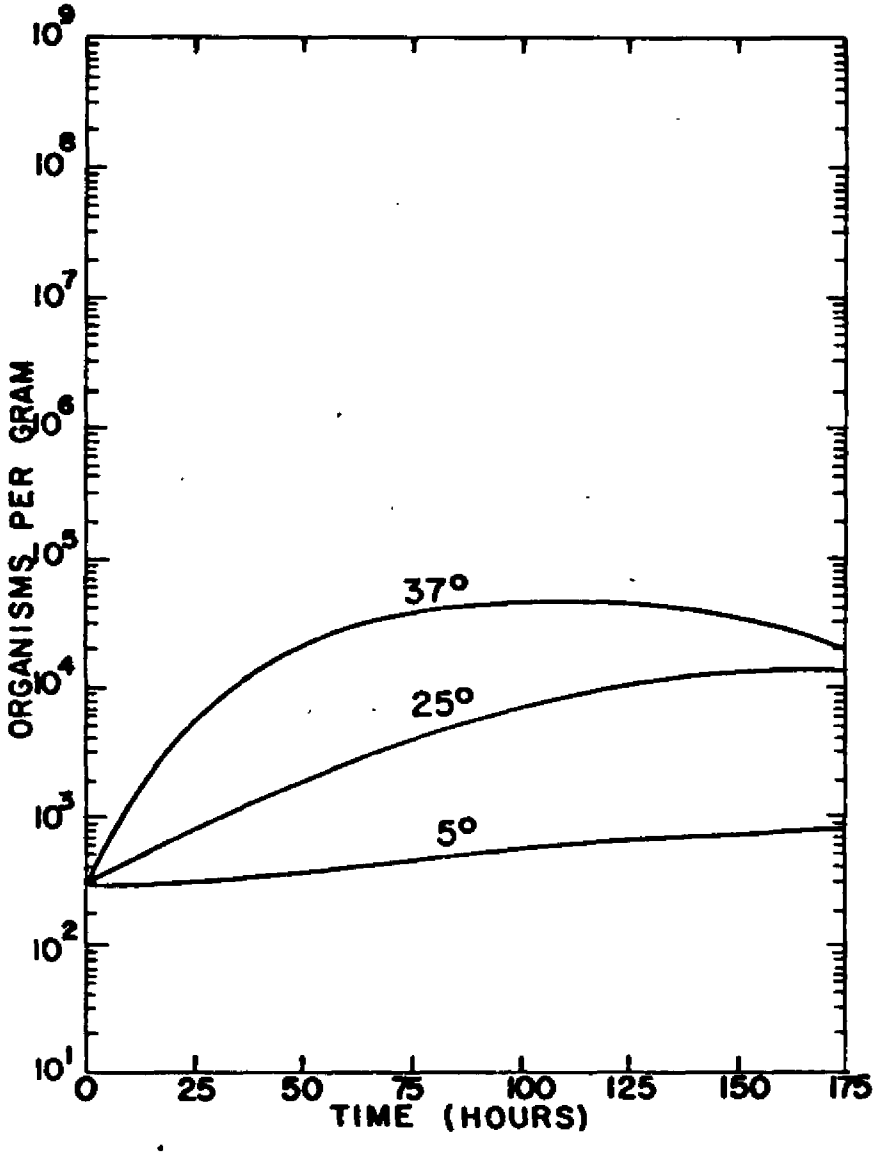

Figure 23. Growth patterns (175-hr) of S. fecalis in raw tailmeat at 5, 25 and 37°C.

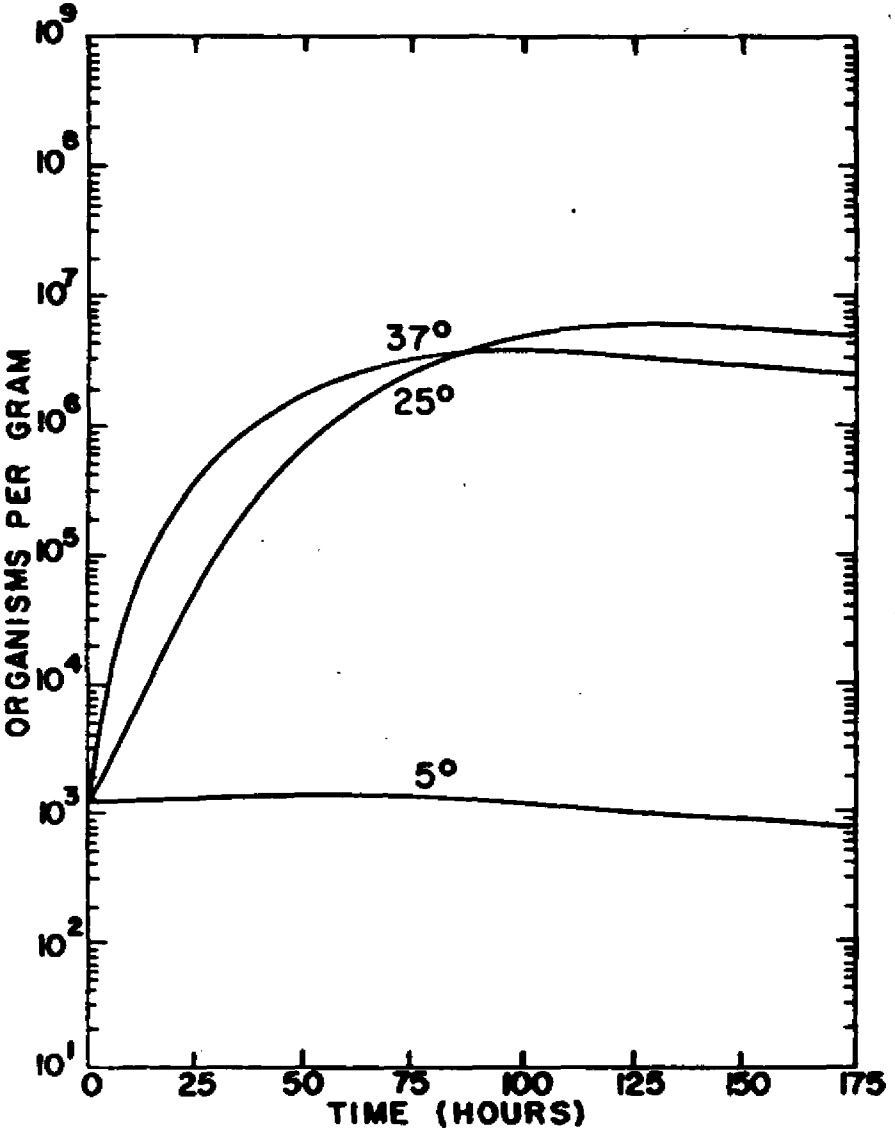

Figure 24. Growth patterns (175-hr) of S. fecalis in cooked tailmeat at 5, 25 and 37°C.

approximately 10^3 to 2 x 10^6 organisms per gram at 72 hr with a very short lag phase. There were no marked changes after 72 hr. At 25°C counts increased from 10^3 to about 10^6 organisms per gram at 72 hr. Counts continued to increase steadily to 6 x 10^6 organisms per gram at 175 hr. At 5°C organisms showed no marked changes during the 175 hr study.

Figure 25 suggests that S. fecalis grew well at 25 and 37°C in sterile crayfish etouffé. At 37°C counts increased from 8 x 10^2 to 10^6 organisms per gram in 48 hr. There was a steady decrease noted throughout remainder of the 175-hr study. At 25°C counts increased from 8 x 10^2 to 5 x 10^6 organisms per gram at 72 hr. There were no marked changes recorded at 175 hr.

Figures 24 and 25 indicate that S. fecalis grew well in sterile crayfish tailmeat and etouffé at 25 and 37°C. However, Figure 23 indicates that its growth was retarded in raw crayfish tailmeat in the presence of other microorganisms and naturally occurring enzymes. Figures 23, 24 and 25 suggest that these three crayfish products do not appreciably affect the behavior of this organism at 5°C for a 175-hr period.

Figures 11 through 25 indicate that several organisms of public health concern are capable of growing well in crayfish products at elevated temperatures. However, at 5°C, all organisms studied with the

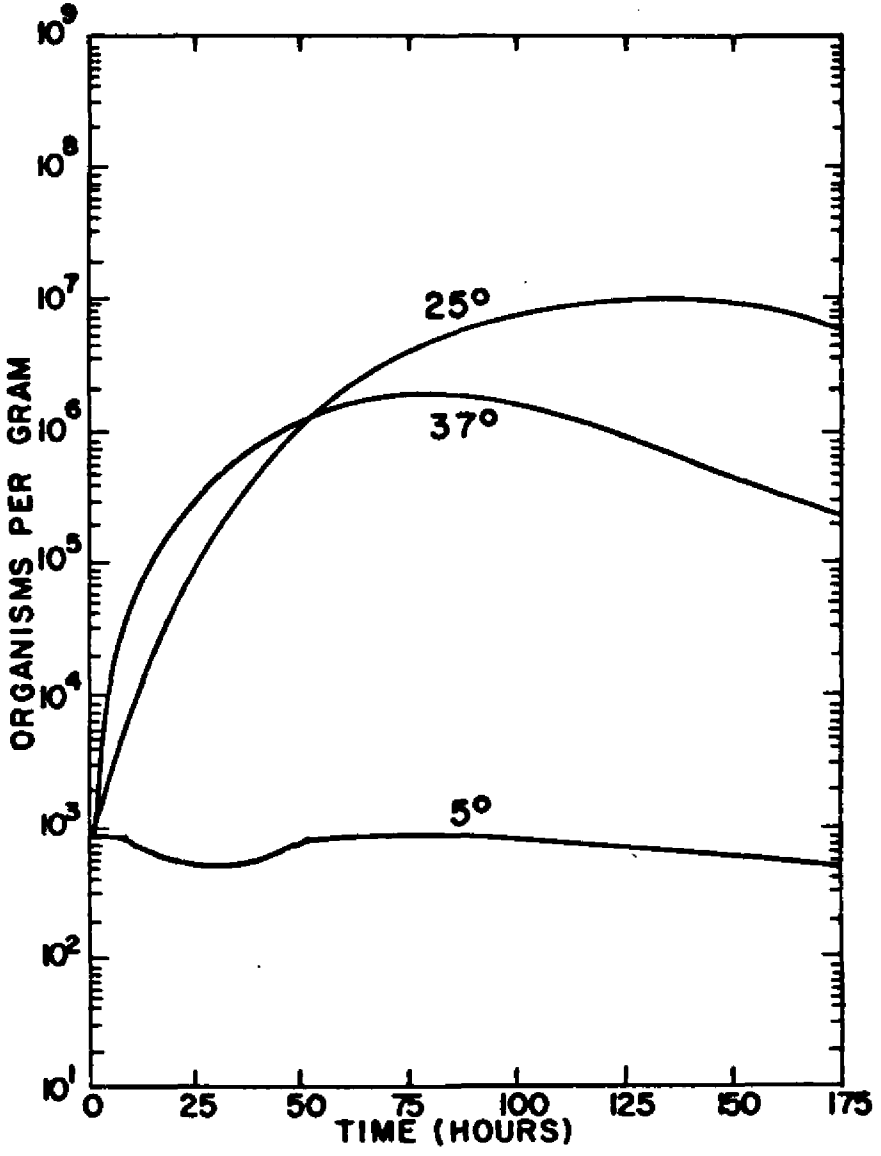

Figure 25. Growth patterns (175-hr) of S. fecalis in etouffé at 5, 25 and 37°C.

exception of S. aureus, in sterile, cooked crayfish tailmeat and crayfish etouffé, do not appear to grow appreciably in a 175-hr period.

IV. Study of Type E Toxin Production by Clostridium Botulinum in Crayfish Products

The purpose of this study was to determine if C. botulinum strain 8E would grow and produce its deadly toxin in raw crayfish tailmeat, sterile-cooked crayfish tailmeat and crayfish etouffé stored at 0, 5 and 30°C for various lengths of time.

Fifty grams of each product was inoculated with 50,000 C. botulinum spores to produce a final concentration of 1,000 spores per gram of sample. Five grams were taken from each sample and a toxin assay was performed. Samples were then placed under anaerobic conditions and stored at 0, 5 and 30°C.

Toxin assay determinations were carried out for 5 consecutive days on products stored at 30°C. According to Pelczar and Reid (1958), C. botulinum grows well from 25 to 35°C.

Table 7 shows results obtained from the 30°C study. In raw tailmeat type E toxin was not produced until 48 hr, however, at 72, 96 and 120 hr toxin was not present. The pH of raw tailmeat when inoculated was 6.7. At 24 hr it had increased to pH 7.0 and no toxin had been produced, however, the product did show evidence of spoilage with a putrid odor and exudation of liquid. At 48 hr the tailmeat was

Table 7. *C. botulinum*, type E toxin production in raw and cooked crayfish tailmeat and etouffé at 30°C. [a]

Product	Criteria	Hours following inoculation 0	24	48	72	96	120
Raw Tailmeat	Toxin	neg.	neg.	pos.	neg.	neg.	neg.
	pH	6.7	7.0	7.3	8.0	8.5	8.5
	Spoilage	no	yes	yes	yes	yes	yes
Cooked Tailmeat	Toxin	neg.	neg.	neg.	pos.	neg.	neg
	pH	6.7	6.9	7.0	7.3	8.1	8.4
	Spoilage	no	yes	yes	yes	yes	yes
Etouffé	Toxin	neg.	neg.	pos.	pos.	pos.	neg. [b]
	pH	6.8	6.7	6.4	6.3	5.9	8.5 [b]
	Spoilage	no	no	yes	yes	yes	yes

[a] Positive toxin production means that 0 mice receiving type E antiserum died, 0 mice receiving heat-treated extract died, and all (2) mice receiving unheated extract died within 24 hours. Any response divergent from this was not considered positive. Spoilage was evaluated "yes" when the product was considered organoleptically unacceptable for consumption.

[b] The pH in etouffé was adjusted to 8.5 by adding 1.0 N NaOH after 120 hours storage.

highly decomposed with a pH of 7.3 and toxin was present. At 72 hr the pH had increased to 8.0 and toxin was not active. The pH continued to rise and the toxin remained inactive.

Toxin was not produced in cooked tailmeat until 72 hr although the product had definitely spoiled within 24 hr. The pH was 7.3 at 72 hr. After 96 hr the pH had increased to 8ml and no toxicity was found. The pH increased to 8.4 after 5 days and the toxin remained inactive.

Toxin was produced in crayfish etouffé at 48 hr and remained present through the 5 day study. The pH ranged from 6.8, initially, to 5.9 after 5 days. These results from raw and cooked tailmeat indicated that at approximately pH 8.0 the toxin was deactivated. In order to confirm this finding the pH of one-half of the etouffé was adjusted to 8.5 with 1 N NaOH on the fifth day of storage and the remainder was left unchanged, and toxin assay procedures were conducted with both samples after 6 additional hours of incubation at 30°C. The sample which was adjusted to pH 8.5 did not show toxin production, whereas the unchanged sample did have the toxin present. Spoilage took place in 48 hr according to odor and appearance. Spoilage may have taken place earlier but spices in this particular product could have masked the odor.

These findings are in agreement with the work of Goldblith and Nickerson (1965). These workers found that in fish press juice, type E,

C. botulinum toxin was destroyed in 29 min at pH 8.0 at 150°F. It was concluded that toxin is much less stable to heat at higher pH levels.

At 30°C all products had spoiled before C. botulinum, type E toxin was produced in crayfish products in this study.

Table 8 shows results obtained at 5°C for raw and cooked tailmeat and etouffé stored for 36 days. Raw and cooked tailmeat as well as the etouffé all showed the presence of the toxin for the first time after 33 days at 5°C. In the raw sample pH increased slowly from 6.7 at 0 days to 7.3 at the time toxin was first noted. At 7 days tailmeat had a good appearance and odor but at 14 days it had deteriorated to a point at which it would be considered unacceptable for consumption. In cooked tailmeat the pH started at 6.7 and increased to 7.0 at the time toxin was first detected. This product was spoiled at 21 days as determined by organoliptically. The pH of etouffé ranged from 6.6 at 0 days to 6.2 at 33 days. This product was judged to be distinctly spoiled at 21 days.

In all three products at 5°C toxin was produced at the same time, 33 days, and spoilage preceded toxin production by at least 12 days. Goldblith and Nickerson (1965) found that Haddock inoculated with 1,000,000 spores per gram of type E C. botulinum became toxic in 8 days when stored at 45°F. Samples inoculated with 10,000 spores per gram became toxic in 16 days.

Table 8. C. botulinum, type E toxin production in raw and cooked crayfish tailmeat and etouffé at 5°C.[a/]

Product	Criteria	Days following inoculation								
		0	7	14	21	24	27	30	33	36
Raw Tailmeat	Toxin	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.
	pH	6.7	6.7	6.8	6.9	7.0	7.2	7.2	7.3	7.3
	Spoilage	no	no	yes	yes	yes	yes	yes	yes	yes
Cooked Tailmeat	Toxin	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.
	pH	6.7	6.7	6.7	6.8	6.8	6.9	6.9	7.0	7.0
	Spoilage	no	no	no	yes	yes	yes	yes	yes	yes
Etouffé	Toxin	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	pos.
	pH	6.6	6.6	6.5	6.5	6.5	6.3	6.2	6.2	6.2
	Spoilage	no	no	no	yes	yes	yes	yes	yes	yes

[a/]Positive toxin production means that 0 mice receiving type E antiserum died, 0 mice receiving heat-treated extract died, and all (2) mice receiving unheated extract died within 24 hours. Any response divergent from this was not considered positive. Spoilage was evaluated "yes" when the product was considered organoleptically unacceptable for consumption.

Table 9 shows results obtained when these samples were stored at 0°C (packed in ice) for 56 days. No toxin was detected in raw tailmeat during the 56-day period, however, the product was judged spoiled at 28 days. The pH ranged from 6.7 at 0 days to 7.5 at 56 days. Toxin was also absent throughout the 56-day study in sterile-cooked tailmeat. This product obtained a rejective odor at 35 days. The pH ranged from 6.7 at 0 days to 7.1 at 56 days.

Toxin was absent, also, over the 56-day study in etouffé stored at 0°C. Spoiled qualities were detected at 42 days, however, this product could have been spoiled earlier with spices masking the adverse odor. The pH ranged from 6.5 at 0 days to 6.2 at 56 days.

This study indicates that C. botulinum strain 8E is capable of producing toxin in raw and cooked crayfish tailmeat and etouffé at 5 and 30°C, but, long after the products have been considered unfit, organoleptically, for human consumption. Perhaps toxin may be produced in these products after 56 days at 0 C, since organisms were capable of causing organoleptic spoilage at 0°C after 28 to 42 days. Fortunately, proteolytic and saccharolytic decomposition of the product rendered them unacceptable for consumption substantially sooner than toxin is produced.

Table 9. C. botulinum, type E toxin production in raw and cooked crayfish tailmeat and etouffé at 0°C.[a/]

Product	Criteria	Days following inoculation								
		0	7	14	21	28	35	42	49	56
Raw Tailmeat	Toxin	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
	pH	6.7	6.7	6.8	6.8	7.1	7.1	7.3	7.5	7.5
	Spoilage	no	no	no	no	yes	yes	yes	yes	yes
Cooked Tailmeat	Toxin	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
	pH	6.7	6.7	6.7	6.7	6.8	7.0	7.0	7.0	7.1
	Spoilage	no	no	no	no	no	yes	yes	yes	yes
Etouffé	Toxin	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
	pH	6.5	6.5	6.5	6.3	6.3	6.3	6.2	6.2	6.2
	Spoilage	no	no	no	no	no	no	yes	yes	yes

[a/] Positive toxin production means that 0 mice receiving type E antiserum died, 0 mice receiving heat-treated extract died, and all (2) mice receiving unheated extract died within 24 hours. Any response divergent from this was not considered positive. Spoilage was evaluated "yes" when product was considered organoleptically unacceptable for consumption.

CONCLUSION

The following conclusions can be stated from results obtained from this study concerning the incidence and growth patterns of some pathogens in freshwater crayfish:

1. Coliforms, E. coli and fecal streptococci are normally found in waters from which commercial catches are obtained. This could be a source of these organisms in the processed products providing improper processing conditions are used.
2. Coagulase-positive staphylococci, Shigella species, Salmonella species and C. botulinum are not normally found in freshwater crayfish or their water environment in south Louisiana. These pathogens when present in processed products will probably be the result of introduction during processing.
3. S. typhimurium, S. dysenteriae, S. aureus, S. fecalis and E. coli do not grow significantly at refrigeration temperatures (ice-packed or at 5°C) in raw and cooked crayfish tailmeat and in etouffé.
4. At 25 and 37°C all of these organisms except S. typhimurium and S. aureus grew well in all of the substrates. S. typhimurium and S. aureus did not show significant growth in raw tailmeat. All of the organisms showed a lower growth rate in raw tissue than in the other products.

5. C. botulinum did not produce type E toxin in crayfish products while stored in ice (0°C) for 56 days.

6. At 5°C it took approximately 33 days for C. botulinum to produce type E toxin in three crayfish products.

7. At 30°C C. botulinum produced type E toxin in raw crayfish tailmeat in 24 hr. Toxin was produced in 48 hr in cooked tailmeat and in etouffé.

8. All products were spoiled at the time toxin was first detected in each case.

9. C. botulinum, type E toxin was inactivated in all these products when a pH of 8.0 to 8.5 was reached. This occurred in trials of raw and cooked tailmeat at 30°C after 2 or 3 days due to metabolism of the microorganisms.

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APPENDIX

A. Difco Liver Veal Agar with 4% Egg Yolk

Fresh eggs were washed with a stiff brush drained and then soaked in 70% ethyl alcohol for 30 minutes. Eggs were cracked aseptically, eggwhites were separated from yolks and discarded leaving the yolk in the eggshell. Yolks were stabbed in the center with a sterile straight inoculating needle opening a hole approximately one fourth inch in diameter. Contents of the yolk sack were taken out by means of an open tip 10 ml pipette and drained into a sterile graduated cylinder. An equal volume of physiological saline was added to the volume of yolk and mixed gently until thoroughly mixed.

Eighty milliliters of egg yolk saline mixture was added to one liter of melted sterile Liver Veal Agar cooled to 45-50 C. Liver Veal Egg Yolk medium was poured into Petri dishes immediately, then dried at room temperature for 2 or 3 days before being used.

B. Trypticase-Glucose-Peptone Medium

1. Trypticase (BBL)	50 g
2. Peptone (Difco)	5 g
3. Sodium Thioglycollate (BBL)	2 g
4. Glucose (Difco)	4 g
5. Distilled Water	1000 ml

Trypticase-Glucose-Peptone Medium was adjusted to pH 7.0 with 1 N NaOH and 1 N HCl and dispensed in 30 ml amounts in 25 x 150 mm screw cap tubes; autoclaved at 121 C for 10 minutes.

C. Trypticase-Glucose-Peptone Agar

1. Trypticase (BBL)	50 g
2. Peptone (Difco)	5 g
3. Sodium Thioglycollate (BBL)	2 g
4. Glucose (Difco)	4 g
5. Agar (Difco)	20 g
6. Distilled Water	1000 ml

Trypticase-Glucose-Peptone Agar was adjusted to pH 7.0 and autoclaved at 121 C for 10 minutes. The media was allowed to cool to 45-50 C and poured into sterile Petri dishes. These were allowed to solidify and dry at room temperature for 24 hours before use.

D. Gel-Phosphate Buffer

1. Gelatin (Difco)	2 g
Na_2HPO_4 (Merck)	4 g
Distilled Water	1000 ml

2. Adjusted to pH 6.2 using 1 N HCl.

3. Autoclaved at 121 C for 15 minutes.

E. Butterfields Phosphate Buffer

The stock solution was prepared by dissolving 34 g of KH_2PO_4 (Merck) in 500 ml of distilled water; adjusted to pH 7.2 with about 175 ml of 1 N NaOH and diluted to 1 liter with distilled water. This stock solution was stored at 5 C. The prepared diluent was ma de by adding 1.25 ml of stock solution per liter of distilled water.

VITA

The author was born in Sulphur, Louisiana on December 4, 1936. Upon graduation from Sulphur High School in May 1955, he entered Northwestern State College in Natchitoches, Louisiana. In August 1959, he was awarded a B.S. degree in Mathematics and a Commission in the United States Army.

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EXAMINATION AND THESIS REPORT

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Major Field: Food Science and Technology

Title of Thesis: Incidence and Growth of Some Pathogens in Freshwater Crayfish (*Procambarus clarkii* Girard)

Approved:

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Date of Examination: May 1, 1967